Aberrant response of human myeloid dendritic cells to microbial stimuli in patients with inflammatory bowel disease

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Dipl. Biochem. Saskia Thomas

Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Dr. h.c. Christoph Markschies

Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I
Prof. Dr. rer. nat. Andreas Herrmann

Gutachter: 1. Prof. Dr. Andreas Radbruch
2. Prof. Dr. med. Daniel C. Baumgart
3. Prof. Dr. med. Hans-Dieter Volk

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A ship in port is safe,

but that is not what ships are for.

Sail out to sea and do new things.

-- Admiral Grace Hopper

Für meine Eltern.

Zusammenfassung

Die Ätiologie der chronisch entzündlichen Darmerkrankungen Morbus Crohn und Colitis ulcerosa ist bis heute ungeklärt. In zahlreichen Studien konnte jedoch an Mausmodellen gezeigt werden, dass dendritische Zellen eine wichtige Rolle im Rahmen der mukosalen Immunabwehr spielen. Eine unkontrollierte Aktivierung von immunologischen Effektorzellen durch antigenpräsentierende Zellen ist die Folge, welche die Antigene der luminalen Flora folglich falsch erkennen und damit letztendlich zu einer Schädigung des Gewebes führen. In der vorliegenden Arbeit wurden humane $CD1c^{+}CD11c^{+}CD14^{-}CD19^{-}$ myeloide dendritische Zellen (mDCs) aus dem peripheren Blut und der intestinalen Mukosa von CED Patienten sowie von gesunden Probanden phänotypisch und funktionell näher charakterisiert.

mDCs von Patienten reagieren auf mikrobielle Modellstimuli wie LPS im Gegensatz zu dendritischen Zellen von Gesunden mit der Ausbildung eines aktivierten Phänotyps und der Sekretion pro-inflammatorischer Zytokine. Die Daten lassen vermuten, dass ihre tolerogene Rolle gestört ist und die Zellen so möglicherweise aktiv zum Entzündungsgeschehen durch eine Fehlreaktion auf die kommensale Flora beitragen. Die TLR4 Expression von mDCs war bei CED Patienten in Remission höher und stieg während eines akuten Schubes weiter signifikant an. Es konnte außerdem gezeigt werden, dass zirkulierende mDCs von Erkrankten mehr LPS aufnehmen. Des Weiteren ist die Häufigkeit von mukosalen und aktivierten mDCs bei CED Patienten im Vergleich zu Gesunden signifikant erhöht. Die vermehrte Häufigkeit von aktivierten mDCs in der entzündeten Mukosa ist ein Hinweis auf intestinales „homing“, also ein Wiedereinwandern der gereiften Lymphozyten in die Darmwand. Die Daten zeigen außerdem eine abweichende LPS Antwort von mDCs bei CED Patienten, die folglich mit einem entzündeten Phänotyp einhergeht.

Es ist bekannt, dass die probiotische Hefe *Saccharomyces boulardii* (*Sb*) eine Wirksamkeit bei entzündlichen sowie infektiösen Erkrankungen des Gastrointestinaltraktes hat. Daher wurde untersucht, ob *Sb* die Funktion von dendritischen Zellen ebenfalls anpasst. Kulturexperimente von humanen mDCs mit Zellkulturüberständen von *Sb* (*Sb*S) und LPS zeigten eine deutliche Reduzierung in der Expression der co-stimulatorischen Moleküle CD40 und CD80 sowie des Reifemarkers CD197 (CCR7) sowohl bei gesunden Probanden als auch bei CED Patienten. *Sb*S reduzierte außerdem die Sekretion zweier wichtiger pro-inflammatorischer Zytokine, TNF-α und IL-6. Während es die Sekretion von IL-10 bei gesunden Probanden erhöhte, konnte bei CED Patienten eine leichte Abnahme verzeichnet werden. Zusammenfassend konnte gezeigt werden, dass *Sb*S die Proliferation von naïven T-Zellen (TCs) in einer

gemischten Lymphozytenreaktion mit gesunden mDCs signifikant vermindert. Ohne Hefe stimulieren mDCs effektiv naïve TCs und führen so zu einer Differenzierung in T_H1- oder T_H2- Zellen. Mittels Membran-Verteilungschromatographie konnte außerdem gezeigt werden, dass die aktive Komponente der probiotischen Hefe ein molekulares Gewicht von weniger als 3 kDa haben muss.

Abstract

Persistence of inflammatory bowel disease is associated with a breakdown of tolerance against the commensal microflora. Various animal studies have provided insights that mucosal dendritic cells play a key role in this process. However, the specific function of certain dendritic cells in IBD is still unknown. Thus, primary $CD1c^{+}CD11c^{+}CD14^{-}CD19^{-}$ myeloid blood (mDCs) and mucosal dendritic cells from IBD patients and healthy controls were compared phenotypically and functionally in this study.

More mDCs from IBD patients exhibited an activated phenotype shown by expression of co-stimulatory molecules as control mDCs. Further, mDCs from IBD patients secrete higher levels of pro- and anti-inflammatory. TLR4 expression by mDCs was higher in remission and significantly increased in flaring UC and CD patients compared with remission and controls. Circulating mDCs from IBD patients take up more LPS and the uptake begins earlier compared with controls. The frequency of mucosal mDCs and the number of activated, i.e. CD40 and CD80 expressing mucosal mDCs, is significantly greater in UC and CD compared with non-IBD controls. The increased frequency of activated mDCs in the inflamed mucosa suggests intestinal homing of mDCs in acute stages of IBD. Further, the data suggests an aberrant LPS response of mDCs in patients suffering from IBD which results in an inflammatory phenotype. The most widely accepted hypothesis for the cause of IBD is a disturbed interaction of the host immune system with commensal microflora and other luminal antigens. The well controlled balance of the intestinal immune system is disturbed and luminal antigens like LPS gain access to the underlying mucosal tissue via the leaky barrier.

Furthermore, it was investigated whether the probiotic yeast preparation *Saccharomyces boulardii* (*Sb*) modulates dendritic cell function which has shown efficacy in inflammatory and infectious disorders of the gastrointestinal tract. Culture experiments of mDCs from healthy volunteers and IBD patients in the presence of *Sb* culture supernatant (*Sb*S) significantly reduced the expression of the co-stimulatory molecules CD40 and CD80 as well as the DC maturation marker CD197 (CCR7) induced by the prototypical microbial antigen LPS. Moreover, *Sb*S reduced secretion of two key pro-inflammatory cytokines TNF-α and IL-6, while the secretion of anti-inflammatory IL-10 increased. However, IBD patients showed also a reduction in their secretion level of IL-10. Finally, *Sb*S inhibited proliferation of naïve T cells (TCs) in a mixed lymphocyte reaction with healthy mDCs. Without the probiotic yeast mDCs effectively stimulate naïve TCs and lead to a differentiation into T_H1 and T_H2 cells. It

could be shown that the active component of the yeast has a molecular weight of less than 3 kDa, as evaluated by membrane partition chromatography.

Schlagwörter:

dendritische Zellen, antigenpräsentierende Zelle, Entzündung, chronisch entzündliche Darmerkrankungen, Morbus Crohn, Colitis ulcerosa, *Saccharomyces boulardii*, T-Zellen

Keywords:

dendritic cells, antigen-presenting cell, inflammation, Inflammatory Bowel Disease, Crohn's Disease, Ulcerative colitis, *Saccharomyces boulardii*, T cells

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Abbreviations

ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
B-cell	Bursa-dependent; bone-marrow-derived (lymphocytes)
BCR	B cell antigen receptor
BM	Bone marrow
BSA	Bovine serum albumin
CBA	Cytometric Bead Array
CD	Crohn’s Disease
cDC	Conventional dendritic cell
cDNA	Copy deoxyribonucleic acid
CDP	Common dendritic cell precursor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DMEM	Dulbecco’s Modified Eagle Medium
DTE	Dithienylethene
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallisable
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
FU	Flare-up
g	Gravitational acceleration
GM-CSF	Granulocyte macrophage-colony stimulating factor
HBSI	Harvey Bradshaw Severity Index
HBSS	Hanks' Buffered Salt Solution
HC	Healthy control

HeBSS	HEPES buffered saline solution
HEV	High endothelial venules
hr(s)	Hour(s)
HS	Human serum
IBD	Inflammatory Bowel Disease
IC	Infectious Colitis
IFN-α	Interferon-α
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilo Dalton
LC	Langerhans cells
LN	Lymph node
LPMC	Lamina propria mononuclear cells
LPS	Lipopolysaccharide
MACS	Magnetic activated cell sorting
MALT	Mucosal-associated lymphoid tissue
mDC	Myeloid dendritic cell
MDP	Macrophage and dendritic cell precursor
MFI	Mean fluorescence activity
MHC	Major histocompatibility complex
min	Minute(s)
MLN	Mesenteric lymph node
MLR	Mixed lymphocyte reaction
MTWSI	Modified Truelove Witts-Severity Index
NK cell	Natural killer cell
ODN	Oligodeoxynucleotides
PAMP	Pathogen-associated molecular pattern
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PI	Propidium Iodide
PMA	Phorbol myristate acetate
pre-DC	Precursor dendritic cell
PRR	Pattern recognition receptor
RM	Remission
RPMI	Rosewell Park Memorial Institute
RT	Room temperature
Sb	*Saccharomyces boulardii*
*Sb*S	*Saccharomyces boulardii* culture supernatant
Sc	*Saccharomyces cerevisiae*
SDS	Sodium dodecylsulphate
SEB	Staphylococcal enterotoxin B
sec	Second(s)
SLE	Systemic lupus erythematosus
TC	T cell
TLR	Toll like receptor
TNF-α	Tumor necrosis factor-α
UC	Ulcerative Colitis
vs	Versus
v/v	Volume per volume

1 Introduction

1.1 The Immune System

Historically, immunity meant protection from disease and more specifically, infectious disease. The cells and molecules responsible for immunity constitute the immune system, and their collective and coordinated response to the introduction of foreign substances is called the immune response. The mammalian immune system can be divided into two different but cooperating compartments – the innate (non-adaptive) and the adaptive (acquired) immune response (Table 1) [1].

Table 1: Major properties of the Innate and Adaptive Immune System.

Property	Innate	Adaptive
Characteristics	antigen non-specific rapid response (minutes) no memory	antigen specific slow response (days) memory
Immune components	natural barriers (e.g. skin) Phagocytes soluble mediators (e.g. complement) pattern-recognition molecules	Lymphocytes (B and T cells) antigen-recognition molecules (B cell and T cell receptor) secreted molecules (e.g. antibody)

Every immune response against a pathogen has different requirements and involves both, appropriate recognition of foreign molecular structures and mounting of an adequate reaction. Thus, both immune responses pursue diverse but complementary defensive strategies and therefore contribute in their own way to successfully resolve an infection.

The innate immune system reacts immediately to invading pathogens [2]. The defence mechanisms of innate immunity range from external physical and biochemical barriers (epithelial cells, mucosal surfaces) to an internal defence (phagocytes, dendritic cells (DCs), natural killer (NK) cells) and also soluble factors such as plasma proteins (i.e. complement cascade, C-reactive protein). Innate immunity is based on a set of germ-line encoded receptors (pattern recognition receptors, PPRs). These receptors are not specific to particular pathogens

but they recognize conserved molecular patterns associated with pathogens (pathogen associated molecular patterns, PAMPs) [3].

In contrast to the innate immune system, there are two types of adaptive immune responses called humoral and cell-mediated immunity, which are accomplished by different components of the immune system. Cell-mediated immunity is arranged by T lymphocytes (T cells, TCs). TCs can efficiently recognize and eliminate infected cells. An important issue of the adaptive TC response is the large diversity of the TC repertoire, which is generated by random rearrangement of gene sequences coding for a functional TC receptor (TCR) during TC development in the thymus. Humoral immunity in the peripheral blood and mucosal secretion is mediated by antibodies that are produced by B lymphocytes (B cells) which mature in the bone marrow (BM). B cells are initially activated to secrete antibodies after the binding of antigens to specific membrane immunoglobulin (Ig) molecules (B cell receptors, BCRs) which are expressed by these cells [4-6]. Once engaged, the B cell receives signals to start the production of the secreted form of this Ig, a process that initiates the antibodies response whose purpose is to eliminate the antigen from the host. B and T cells differ in many functional aspects but share one of the important properties of the immune response – they exhibit specificity toward an antigen. Thus the major recognition and reaction functions of the immune response are contained within the lymphocytes.

Indeed, the high degree of diversity might lead to a reaction against endogenous structures of the organism. Therefore, sophisticated mechanisms are required to ensure tolerance within the immune system. Thus, the prime task of the immune system is to sustain the subtle balance between immunity and tolerance. A failure of mechanisms that control central and peripheral tolerance can lead to autoimmune diseases like type 1 diabetes, lupus or rheumatoid arthritis (RA) [7]. The defining characteristics for adaptive immunity are exquisite specificity for distinct molecules and the ability to remember and respond more quickly and efficiently to repeated infections (immune memory) [8-10].

1.2 Dendritic Cells

DCs are professional antigen-presenting cells (APCs) that play a central role in the immune system. They were first described in the human skin by Paul Langerhans in 1868, who assumed that they were cells of the nervous system based on their morphology and finally called them Langerhans cells (LCs) [11]. However, the discovery of a small population of "large stellate" cells in peripheral lymphoid organs of mice in 1973 by Steinman and Cohn reinitiated the modern research of DCs [12;13]. It could be established that LCs as well as DCs in the skin and the thymus are not nerve cells but originate from hematopoetic progenitors of the BM [14-16]. They are the most important cell type responsible for antigen presentation. As APCs they control the initiation and the maintenance of adaptive immune responses, as well as the induction of peripheral tolerance [17-20].

1.2.1 Subsets of human dendritic cells

DCs make a rare cell population within the human body. They constitute only 1-3% of all skin cells and 0.2% of peripheral blood mononuclear cells (PBMCs) [21]. Due to their low frequency and the absence of specific antibodies it was long very difficult to identify, isolate and enrich these cells. In 1994 Romani et al. were the first who successfully isolated DCs from human blood [22].

DCs present a heterogenous population of specialized BM derived leukocytes. They are derived from multiple lineages and have distinct stages of cell development, activation and maturation. Further, DCs feature the potential to induce both immunity and tolerance [13;23]. Under steady-state conditions DCs exist as either conventional (cDCs) or precursor DCs (pre-DCs). Different subsets of DCs are described in human peripheral blood. However, the majority of DC subsets are of myeloid origin and they are generated in large numbers in the BM every day. These cells are also termed $CD11c^+$ DCs, DC1 or myeloid DCs (mDCs) and express on the one hand high levels of the granulocyte macrophage-colony stimulating factor (GM-CSF) and on the other hand low levels of the IL-3 receptor (CD123). These progenitor cells initially transform into immature DCs and are characterized by a high endocytic activity and with a low potential for TC activation. The second major subset of DCs in human population express high levels of CD123 but little GM-CSF receptor and need to be activated before displaying characteristic stimulatory activity *in vitro*. They are also called $CD11c^-$ or

plasmacytoid DCs (pDCs). Subsets of DC precursors circulate in the blood stream and migrate into tissues, where they reside as sentinels in an immature state [24].

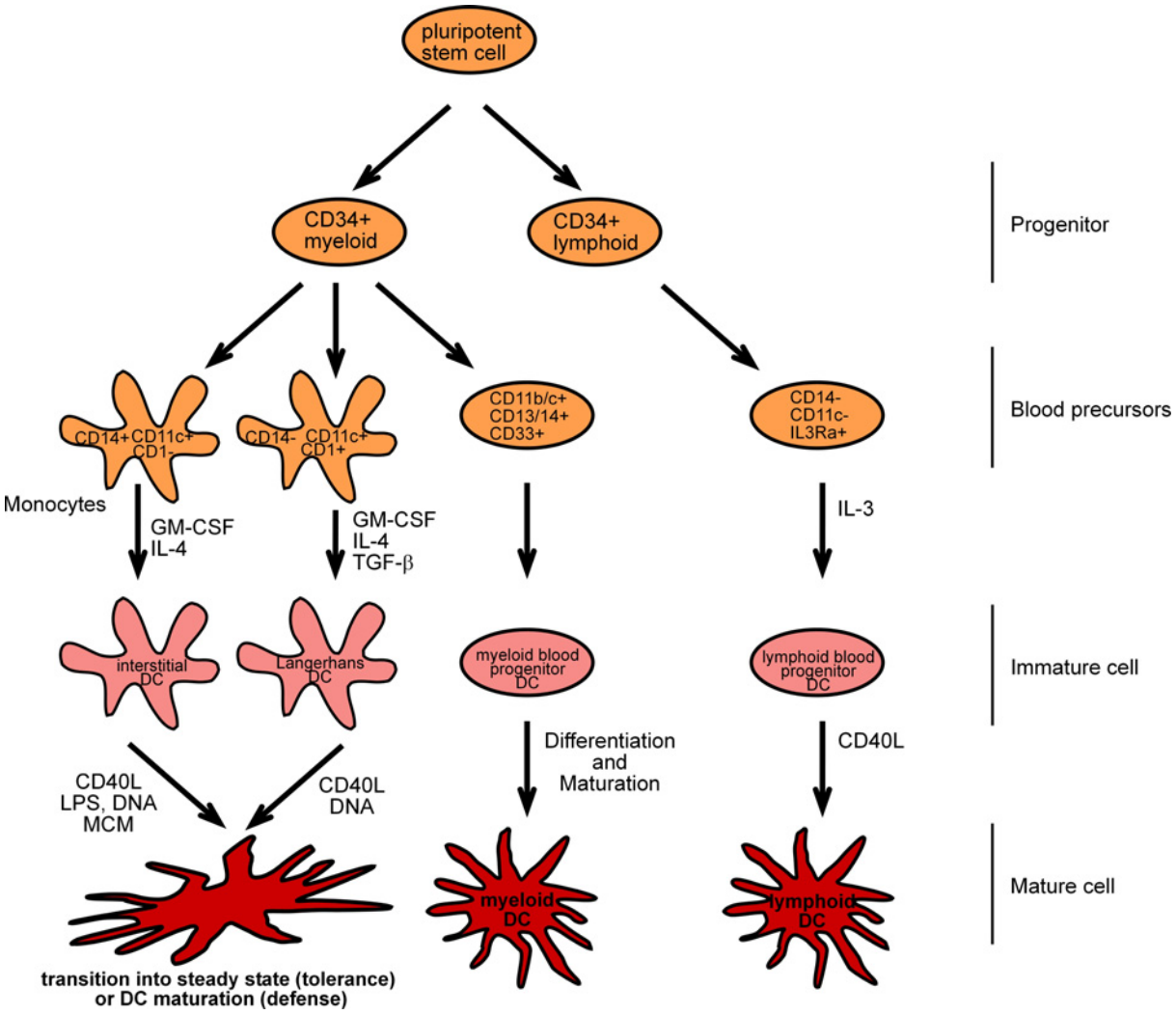

Figure 1: Subsets of human dendritic cells. Myeloid progenitors differentiate into monocytes which are $CD14^+CD11c^+CD1^-$ DC precursors that yield to immature DCs in response to granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4. On the other site myeloid progenitors can also differentiate into $CD14^-CD11c^+CD1^+$ precursors which yield to LCs in response to GM-CSF, IL-4, and transforming growth factor-β (TGF-β). A second lymphoid-related DC differentiation pathway can be described, that give rise to plasmacytoid DCs (pDCs) where $CD14^-CD11c^-IL3R\alpha^+$ DC precursors can originate from the lymphoid $CD34^+$ progenitor [25;26]. The $CD14^-CD11c^-IL3R\alpha^+$ precursor cells differentiate into immature DCs in response to IL-3. All immature cells finally differentiate into mature cells in response to cytokines or pathogen products. (Adapted from Banchereau et al, 2000 [26])

Recent work unveiled new aspects of the developmental and lineage relationships among DC populations [27]. However, this unique work by Liu and colleagues is limited on *in vivo*

experimental approaches in the mouse. The authors were able to show that DC development progresses from the macrophage and DC precursor (MDP) that is indentified by its surface phenotype (Lin^- $cKit^{hi}$ $CD115^+$ CX_3CR1^+ $Flt3^+$) to common DC precursors (CDPs). CDPs no longer rise to monocytes but they can produce pDCs and classical (conventional) spleen DCs (cDCs). Finally, they rise to pre-cDCs and commit to cDC development. Furthermore, intravital imaging studies indicated that pre-cDCs emerge from the BM, travel through blood and finally reach the lymph node (LN) through high endothelial venules (HEVs). In the first instance pre-cDCs migrate along HEVs and later disperse throughout the LN TC area to finally integrate into the DC network. The origin and differentiation cues for many tissue macrophages, monocytes, and DC subsets in mice, and the corresponding cell populations in humans, remain to be elucidated.

1.2.2 Dendritic cell maturation and migration

DCs are leukocytes, distributed throughout lymphoid and non-lymphoid tissues, in peripheral blood and afferent lymph vessels. The main function of immature DCs is the uptake of exogenous antigens in the periphery. They constantly sample their environment for antigens at potential sites of pathogen entry. This is done through receptors that recognize specific chemical signatures which are found on subsets of pathogens. DCs express numerous receptors like toll-like receptors (TLRs) that recognize conserved structures, known as PAMPs that are unique to the microbial world and found on entire classes of pathogens (i.e. bacterial LPS or double-stranded viral RNA) [28]. Several C-type lectin and lectin-like receptors have been characterized that are expressed abundantly on the surface of these professional APCs [29]. Furthermore, DCs express Fc-receptors which bind to antibody-opsonising particles or cells as well as receptors of the complement system [30].

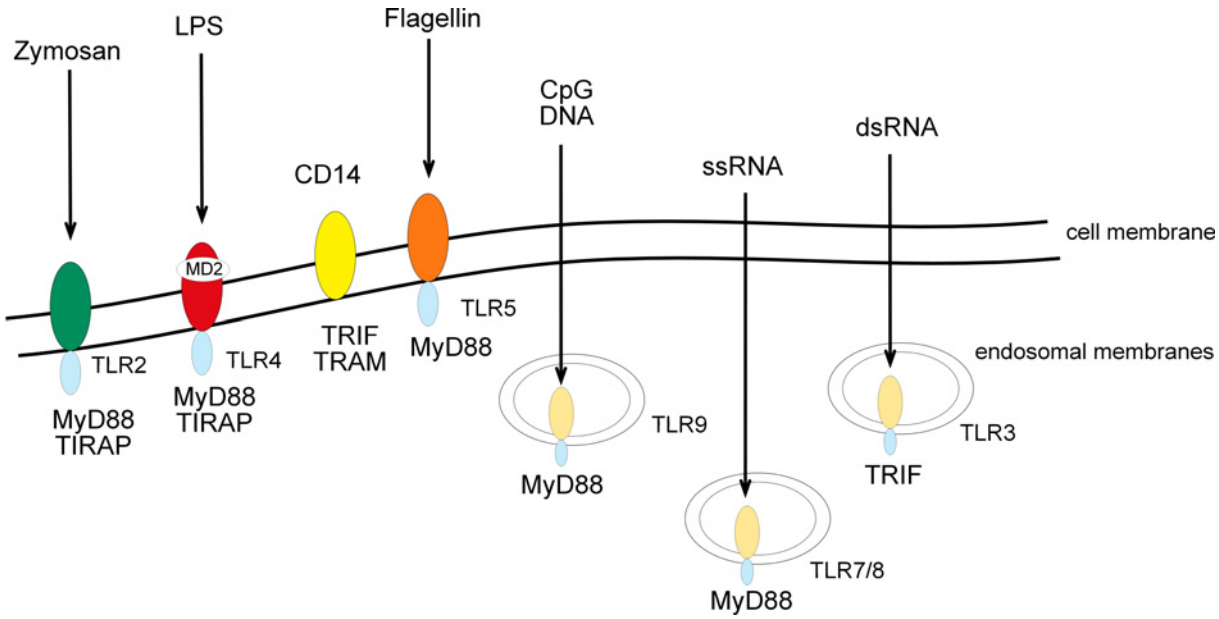

Figure 2: Toll-like receptors and their ligands. The family of TLRs consists of 13 members. Each individual receptor recognizes viral and/ or bacterial components and initiates the activation of immune cells. TLR2, 4, and 5 expressed on the cell surface recognize either zymosan, bacterial LPS and flagellin, respectively. Nucleic acids mainly originating from viral patghogens like CpG DNA, dsRNA and ssRNA are recognized by TLR3, 7/8 and 9. They are localized intracellular and detect nucleic acids in compartments that are normally not accessible to the nucleic acids derived from the host.

Upon encountering an antigen and the exposure to so-called 'danger' signals like pathogens, tissue damage, and local inflammation, immature DCs migrate to lymphoid organs (i.e. LN). During this process, which is regulated by an altered expression of chemokine receptors, DCs undergo maturation. Immature DCs phagocytose pathogens and degrade its proteins into small pieces and upon maturation present those fragments at their cell surface using MHC molecules. When TLR ligands bind their receptors, an intracellular signalling cascade induces DC activation and maturation as defined by an up-regulation of MHC class I and II, an increased expression of co-stimulatory molecules and the secretion of specific cytokines [31]. It could be shown that human DC subsets express distinct patterns of TLRs and may subsequently be suited to encounter different pathogens and finally regulate immune responses [32;33]. That process is characterized by a down-regulation of the capacity to capture antigen and an up-regulation of antigen processing and presentation, as well as an up-regulation of co-stimulatory molecules which act as co-receptors in TC activation greatly enhancing their ability to activate TCs.

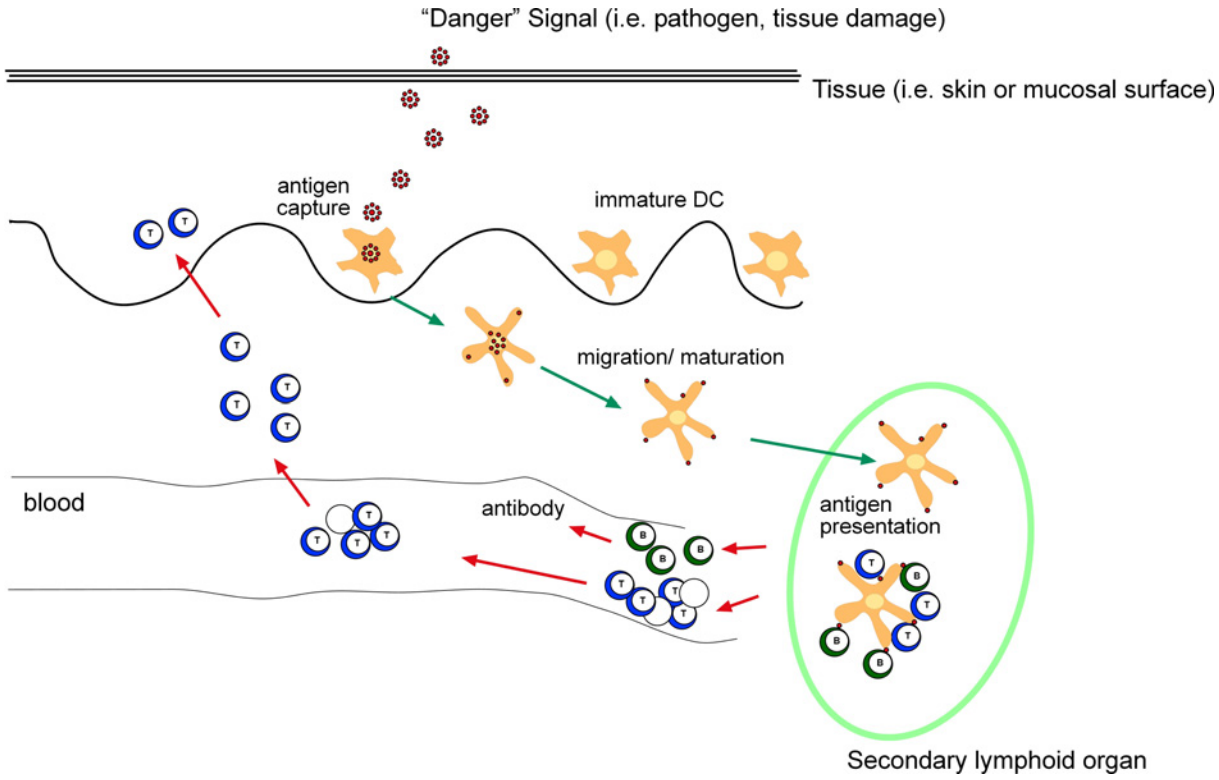

Figure 3: Life cycle of a dendritic cell. DCs originate from bone marrow-derived $CD34^+$ progenitors and finally migrate into tissues all over the body. DC progenitors reside in tissues in an immature state. Invading pathogens are captured by immature DCs followed by antigen processing. Consequently, DCs mature and migrate to secondary lymphoid organs where they present the antigen to TCs and initiate an immune response. Activated TCs finally migrate to the site of inflammation whereas B cells become activated after the contact with TCs and DCs migrate into various areas in the body. Here, B cells mature into plasma cells which are secreting antibodies that can neutralize the invading pathogen. B – B cell, DC – dendritic cell, T – T cell.

pDCs are sparsely distributed and found in blood and lymphoid tissues (i.e. thymus, tonsils, spleen) [25;34-37]. mDCs which are widely distributed are also found in peripheral blood. Mature DCs are a final stage of differentiation, and they cannot be converted into either macrophages or lymphocytes. All DC populations show a characteristic process of maturation which leads to phenotypic and functional change.

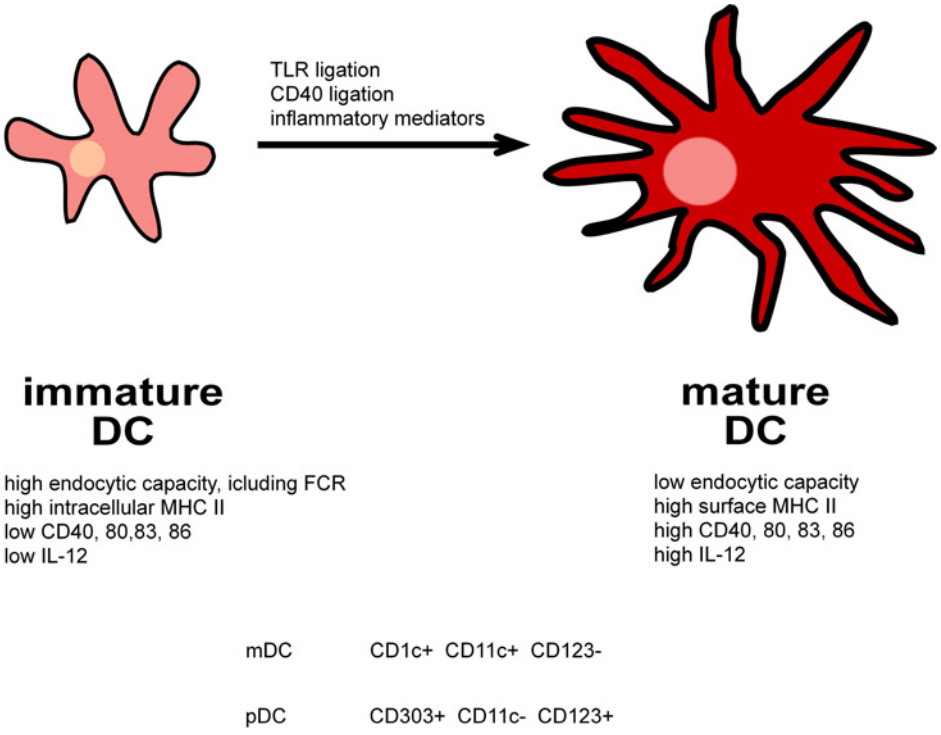

Figure 4: Phenotypic and functional properties of immature and mature dendritic cells. Features like phenotype and function change during DC maturation. Immature DCs take up antigen i.e. via phagocytosis, macropinocytosis or adsorptive pinocytosis. DC – dendritic cell, IL – interleukin, mDC – myeloid DC, pDC – plasmacytoid DC. (Adapted from Banchereau et al, 1998 [13])

Functionally, DCs exert various effects on other immune cells, particularly in secondary lymphoid organs. Here, they present non-self peptide-MHC complexes to naïve and memory T lymphocytes to mobilize specific immunity. The capacity of DCs to induce stimulatory TCs is dependent on the state of DC maturation. By contrast, in order to induce TC tolerance in the thymus, DCs present self peptide-MHC complexes to thymocytes. Tolerance is induced in the absence of inflammatory stimuli when immature, resting DCs are very poor stimulators for the proliferation of resting TCs. During that process DCs are not activated and they are not able to express any co-stimulatory molecule. The capacity of DCs to initiate primary immune responses is due to their ability to deliver specific co-stimulatory signals which are essential for TC activation from the resting or naïve state into distinct classes of effector cells. These immunogen-specific immune responses are critical for tumor resistance, prevention of metastasis, and blocking infections.

In addition, DCs play an important role in innate immunity by secreting cytokines, i.e. IL-12 and type I and II interferons which are involved in host defense mechanisms. Moreover, they

activate NK cells which rapidly eradicate selected targets. Therefore, additional pro-inflammatory signals are necessary for the activation of DCs. Immature DCs express different receptors on their surface that are specific for pro-inflammatory cytokines (i.e. TNF-α, IL-1β, IL-18) [38]. A lack of these signals lead to a release of anti-inflammatory cytokines such as IL-10 or TGF-β by immature DCs. Consequently, TCs are being inactivated and regulatory TCs are differentiated which are able to protract or even avoid an immune response [39].

A third known mechanism for the activation of DCs is mediated by direct cell-cell contact and the interaction of the surface receptor CD40 on DCs with the CD40 ligand (CD40L) on activated TCs [40]. For an ideal initiation of DC maturation the presence of several signals is necessary.

1.2.3 Dendritic cell mediated activation of naïve T cells

DCs are cells of the innate immune system and are acutely activated when a pathogen invades the body, but they also play a decisive and instructive role in the adaptive immune response that arises [41]. Given that the induction of productive TC responses depends upon activation of DCs, it follows that the DC serves as a pivotal interface bridging the innate and adaptive immune system. After delivery of their pathogen-related information, DCs are no longer necessary for the acute response to antigen, presumably they undergo apoptotic cell death and are eliminated.

In the secondary lymphoid organs, mature DCs present antigens captured in the periphery to resting or naïve TCs, inducing an adaptive immune response. Therefore, the TCR binds the presented antigen and the two cells form an immunological synapse from DCs with the according ligands of the TC. The activation and clonal expansion of naïve TCs depends on three independent signals. Without these signals the TC becomes anergic and shifts into a non-proliferating state. The first signal is provided by binding of the TCR receptor to a short peptide presented by MHC on the DC. This ensures that only TCs with a TCR specific to that peptide are activated. The second signal comes from co-stimulation, in which surface receptors on the DC are induced by a relatively small number of stimuli, usually products of pathogens, but sometimes breakdown products of cells, such as necrotic-bodies or heat-shock proteins. The only co-stimulatory receptor expressed constitutively by naïve TCs is CD28, so co-stimulation for these cells comes from the CD80 and CD86 proteins on the DC [42]. This

signal is intensified by an additional interaction of CD40 and CD40L [40]. The combination of these two signals induces the expression of IL-2 and his receptors in the TC. The autocrine linkage of IL-2 leads to an expansion of the antigen-specific TC [43]. Other receptors are expressed upon activation of the TC, such as OX40 and ICOS, but these largely depend upon CD28 for their expression. Finally, a third signal is necessary to completely activate naïve TCs. Therefore, DCs and other immune cells secrete specific cytokines [44]. Important for the differentiation of $CD4^+$ TCs to T_H1 cells are IL-12, IFN-γ and IFN-α whereas the attendance of IL-4 promote a differentiation in T_H2 cells [45;46]. The proliferation as well as the cytotoxic activity of $CD8^+$ TCs is stimulated by signalling of IL-15 and other interferons [47;48].

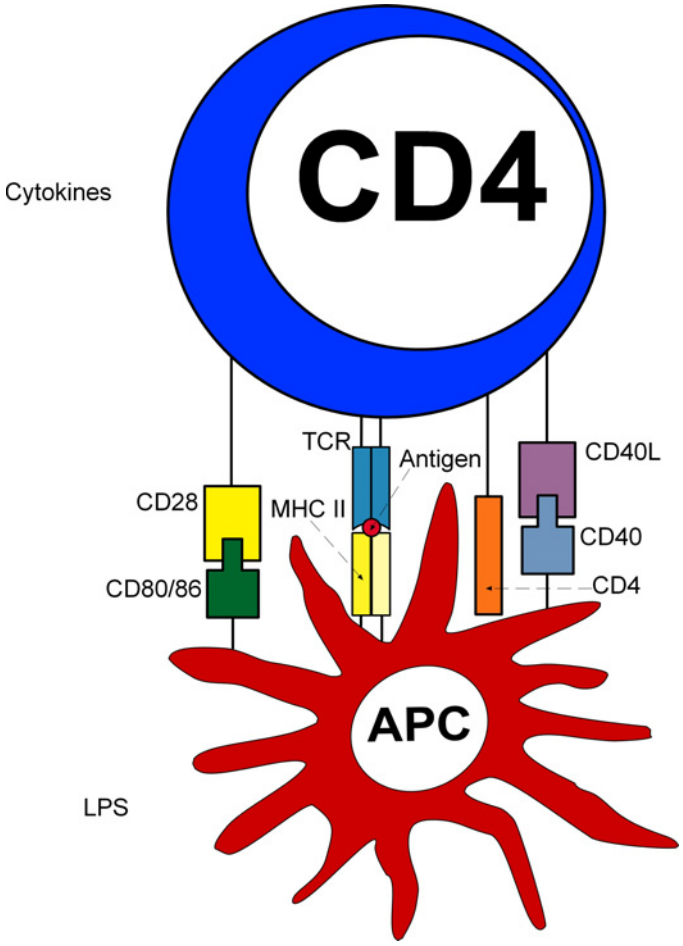

Figure 5: Signals for T cell activation. For the activation of TCs specific signals are necessary. Signal 1 is the antigen-induced signal which is delivered to the TC by the TCR-peptide-MHC interaction. $CD4^+$ TCs recognize the antigen in association with MHC II molecules. Signal 2 is conducted through the co-stimulatory CD28 on the TC which binds to CD80 (B7.1) and/ or a binding to CD86 (B7.2) on the APC. The CD40L-CD40 interaction displays an additional signal for $CD4^+$ TCs. Cytokines as well as other exogenous factors like LPS provide the third signal for the activation of naïve TCs. APC – antigen-presenting cell, TCR – T cell receptor, TC – T cell.

DCs are the most important cell type responsible for antigen presentation, and they control both the initiation and maintenance of adaptive immune responses, as well as the induction of peripheral tolerance [17-20].

1.3 The gastrointestinal immune system

The intestinal mucosa implies a large number of mononuclear cells, including macrophages and DCs. They are all together believed to play a central role in regulating mucosal adaptive and innate immune responses [49]. The gut contains the largest proportion of the mucosa-associated lymphoid tissue (MALT) in the organism. Here one can find more lymphocytes than in any other lymphatic organ. The lymphatic tissue of the gut covers TCs, B cells, granulocytes, mastocytes, macrophages and DCs [50;51].

The intestinal tract encounters an enormous load of antigens while coevally maintaining a normal homeostatic environment. Most antigens are beneficial to the host, such as dietary antigens and symbiotic bacteria, and some are harmless like commensals. All together the intestine harbours the largest and most diverse microbiota which consists of more than 500 species of bacteria [52;53]. On the other hand, pathogens need to be recognized and eliminated before damage occurs. Whereas the systemic immune system elicits an aggressive immune response to exposure of any non-self antigen, the intestinal immune system needs to be more flexible. Antigens need to be sampled, processed, and presented in a way that allows the elimination of pathogens and tolerance to nonpathogens. The challenge of facing billions of bacteria, limitless dietary antigens, and the largest pool of lymphocytes in the body necessitated the development of unique cells as well as mediator and regulatory processes [52;53]. Cells of the gut-associated lymphoid tissue (GALT) include conventional cells of the innate and adaptive immune system (i.e. B and T lymphocytes, macrophages, DCs), non-classical APCs, such as intestinal epithelial cells (IECs), and finally lymphocytes specific for the GALT, called lamina propria lymphocytes (LPLs) and intestinal epithelial lymphocytes (IELs).

1.3.1 Barrier and unspecific defense mechanisms

Mucosal surfaces are physical interfaces between the immune system and the massive antigenic load represented by the commensal and potentially pathogenic enteric bacteria [54]. A variety of mechanisms contribute to the ability of the gut to either react or remain tolerant to antigen present in the intestinal lumen. The epithelial cell layer forms a barrier against exposure to mucosal microflora and other mucosal antigens. Thus it plays a key role in the regulation of mucosal immune responses. Crucial for an efficient barrier function are specialized adaptations of the intestine, including mucus secretion, tight junctions between epithelial cells, defensins and IgA. Mucus is produced as a thick layer along the intestinal membrane and it offers numerous functions like trapping bacteria and viruses, preventing them from gaining initial access to the host and serving as a microenvironment for the accumulation of bacteriocidal and bacteriostatic chemical enzymes. Furthermore, IECs form intercellular tight junctions that effectively restrict transepithelial movement of particulate and even hydrophilic molecules. Therefore, they prevent an uncontrolled uptake of bacteria and many of their metabolites [55]. However, the defense function of the intestinal epithelium is not limited to providing a barrier. Instead, the intestinal epithelium actively interacts with microbes and immune cells by secretion of cellular immune mediators.

1.3.2 The intestinal immune system in healthy state

The intestinal immune system represents a complex network of lymphoid and non-lymphoid cells as well as humoral factors. In contrast to DCs from the systemic immune system, mucosal DCs seem to preferentially induce regulatory TCs [56-59]. These properties of the mucosal immune system might be due to the high concentration of anti-inflammatory cytokines, such as TGF-β, IL-4 and IL-10. Luminal antigens are sampled by DCs. Uptake and presentation of antigens to naïve T and B cells to induce an adequate immune response is the primary function of the M cells in the mucosa. Following ingestion, antigens and microorganisms are transported from the gut lumen to the dome region through these specialized M cells. In place they encounter APCs leading to typical interactions between DCs and naïve TCs (T_H0) through MHC II receptors. In the healthy intestinal immunes system, immature DCs control not only the adaptive immune response, like the balanced differentiation of naïve TCs into effector TCs (T_H1, T_H2, T_H17) that are necessary to

antagonize pathogens and induce regulatory TCs (T_{reg}, T_H3), but also the innate immune response that provides activation of NK cells. T_H3 cells, a population of $CD4^+$ TCs that produce transforming growth factor-β (TGF-β) can be generated by repeated restimulation of mesenteric lymph nodes (MLNs) or splenic lymphocytes from mice that have been fed low dose of antigen for oral tolerance induction. In the presence of commensals and the absence of inflammation in this area, a balance between effector and regulatory immune cells is maintained by a complex and controlled cytokine network. In the presence of cytokines like IL-12 and IFN-α TCs can differentiate into T_H1 cells, whereas IL-4 can induce the differentiation into T_H2 cells. T_H1 cells express the IL-12 receptor β2 chain and the IL-18 receptor, whereas T_H2 cells express an IL-1-like molecule that appears to regulate T_H2 effector functions both in the peripheral and mucosal immune system [60]. After induction into the GALT, mature lymphocytes leave the inductive sides and finally migrate to the effector sides such as the lamina propria. Here they can induce pro-inflammatory as well as suppressive immune responses. Among the pro-inflammatory signals mucosal T_H1 and T_H2 effector cells also produce cytokines that have a central regulatory role in the immunhomoeostasis.

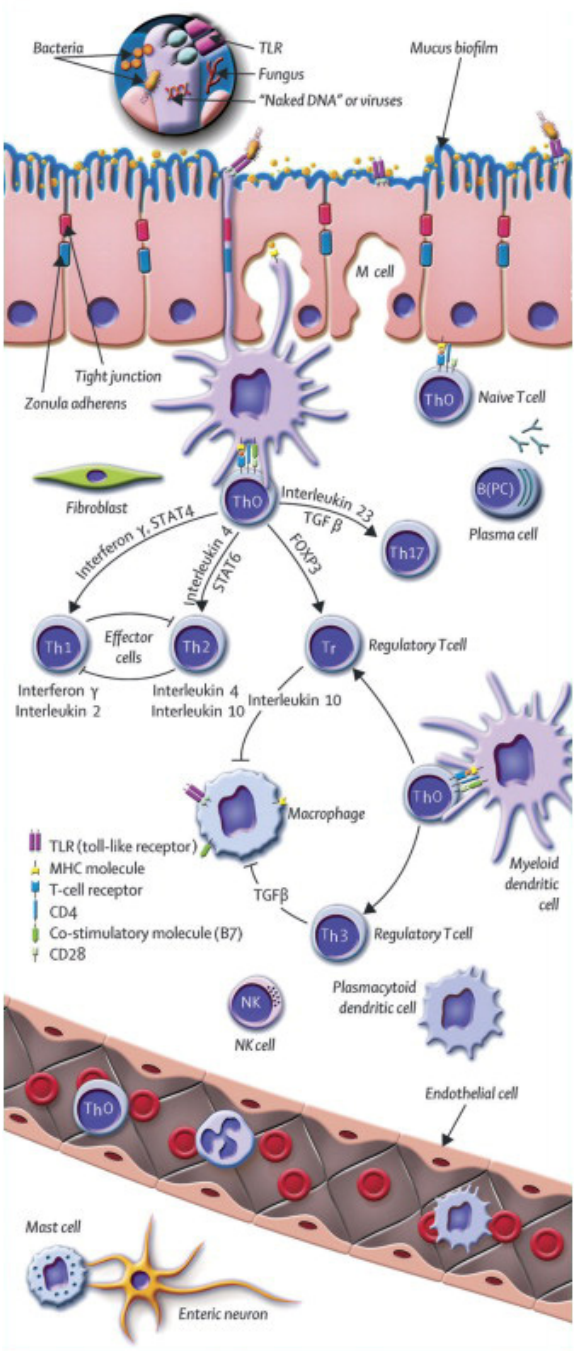

Figure 6: The intestinal immune system in healthy state. Antigen can pass the epithelium via M cells and after transfer to local DCs it might directly be presented to naïve T_H0 cells. T_H0 cells direct further differentiation by the production of different cytokines. Under healthy conditions, a balance between the generation of pro-inflammatory T_H1/ T_H2 and anti-inflammatory T_r/ T_H3 cells is established. After the activation the cells spread widely via the lymphatic system and finally reach the lamina propria and the epithelium of the intestine. M cell – microfold cell; TC – T cell; T_H – T helper cell; T_H0 cells – naïve T cell; T_H, T_H1, T_H2, T_H17 cells – effector TC; T_r, T_H3 – regulatory T cells; B – B cell; B(PC) – plasma cell; NK – natural killer T cell. (Adapted from Baumgart et al, 2007 [61]).

1.3.3 Cytokine regulation of the mucosal immune response via mucosal T cells

Antigens can pass the mucosal epithelium through M cells and finally encounter with local DCs which direct the differentiation of naïve $CD4^+$ T_H0 cells to one of the several states of polarized cytokine production under the influence of cytokines and their associated signaling pathways [62].

T_H1 cells secrete pro-inflammatory cytokines like IFN-γ, IL-2, and TNF-α whereas T_H2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, and promote expression of IgA and other Ig isotypes. Moreover, T_H3 cells are also known to secrete TGF-β whereas regulatory TCs predominantly produce IL-10. Many of the TC functions in the gastrointestinal immune system are mediated by secreted cytokines. Previous studies have shown that lamina propria TCs produce higher levels of IL-10 compared to peripheral blood lymphocytes [63;64]. This indicates an inhibition of T_H1 activation by IL-10 secreting regulatory TCs. Furthermore, the production of IL-10 in the intestine acts on macrophages to prevent their activation and the induction of pro-inflammatory cytokines, thereby inhibiting the recruitment of TCs to the intestine. Other studies demonstrated that regulatory TCs, induced by oral antigen uptake, have characteristics of T_H2 or T_H3 cells [65;66]. On the other hand, work with TCR transgenic mice showed that continuous feeding of low dose antigen induces a T_H1 response [67]. However, there is also data indicating that T_H1 cytokine production may not only have a pro-inflammatory effect as it also can be protective in the immune regulation of certain infectious or autoimmune diseases [68].

1.3.4 Oral tolerance

The ability of the mucosal immune system to distinguish between harmful and harmless antigens is essential to establish highly protective immune responses and further prevent the induction of mucosal pathology. One mechanism that inhibits reactive immune responses is the induction of oral tolerance. Oral tolerance is defined as the induction of a state of systemic immune unresponsiveness to orally administrated antigen upon subsequent antigen challenge such as the absence of antigen-specific TCs. This mechanism presumably prevents the development of an immune reaction or allergy against intestinal intraluminal antigens. However, the mechanisms responsible for establishing and primarily maintaining oral

tolerance to the microbiota and food-derived antigens are not yet defined. They involve a complicated interplay of anatomical, cellular as well as humoral factors that prevent immunity against antigens that approach from the lumen. Otherwise the intestinal lumen would trigger an inflammatory response, when presented to the immune system by a non-oral way [69].

The mucosal immune system matures during microbial colonisation meanwhile immune or oral tolerance is established.

1.4 Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is a chronic relapsing, immunologically mediated disorder of the gastrointestinal tract. There are two main forms of IBD - ulcerative colitis (UC) and Crohn's disease (CD) - which have many similarities and also several clinical and pathological differences. IBD leads to long-term and in some cases to an irreversible impairment of the gastrointestinal function and structure [70;71].

1.4.1 Crohn's Disease

CD was first recognized by the German surgeon Wilhelm Fabry in 1623 [72]. Later, in 1932 it was described and named after the physician Burril B. Crohn [73]. It primarily causes abdominal pain, diarrhea, vomiting, and weight loss. It may affect any part of the gastrointestinal tract, but most commonly the disease starts at the terminal ileum [74].

1.4.2 Ulcerative Colitis

UC was first described by the British physician Sir Samuel Wilks in 1859 [75]. The main symptoms in active UC are diarrhea mixed with blood and mucus, of gradual onset [76]. In 50-70% the disease is limited to the rectum and sigmoid colon [77]. Sometimes it is limited to the rectum as ulcerative proctitis.

1.4.3 Epidemiology

Both diseases have a prevalence range of 10-200 cases per 100,000 individuals in Europe and North America and are becoming more common in the rest of the world as different countries adopt a western lifestyle [78]. These observations indicate that there are strong environmental influences on IBD. This was already confirmed in twin studies by the relatively low concordance rate in identical twins, ~50% for CD and ~10% for UC [79].

1.4.4 Etiology and pathophysiology

Although the exact etiology remains uncertain, it is hypothesized that IBD results from inappropriate and ongoing activation of the mucosal immune system which is driven by the presence of the normal luminal flora. This aberrant response is most likely facilitated by genetic prepositions and defects the barrier function of the intestinal epithelium as well as the mucosal immune system.

1.4.5 Genetic factors

It has been previously shown in mouse models that genetic defects can lead to the development of spontaneous mucosal inflammation. Genetic studies highlight the importance of host-microbe interactions in the pathogenesis of IBD [70;80-83]. Entirely different genetic abnormalities can lead to similar features of the inflammation in the intestine. IBD is a complex polygenic disease with differential concordance rates in twins. In CD, monozygotic twins have a concordance rate of 58%, while dizygotic twins have rates similar to siblings. On the other hand, in patients with UC the concordance rates are lower than in its counterpart (monozygotic twins, 6-17%; dizygotic twins, 0-5%) [84-87]. Furthermore, it could be demonstrated that 20% of patients suffering from CD carry mutations in the *nod2/card15* gene, which is involved in the regulation of host response to bacteria [88]. CARD15 is an innate immunity receptor which has been strongly associated with the pathogenesis of CD. Homozygotes carrying two risk mutations and have a 44 times greater probability of contracting CD than noncarriers of the mutations [89]. Another important genetic factor is chromosome 6 that has been implicated in the etiology of IBD. Work from several groups previously reported associations that include class I, class II, and tumor necrosis factor-a

(TNF-α) alleles [90-94]. Alltogether, these facts illustrate that genetic changes can cause colonic inflammation.

1.5 Malfunction of the immune system in Inflammatory Bowel Disease

The well controlled balance of the intestinal immune system is disturbed at all levels in IBD. The immunological nature of the disease arises from the observation that IBD is characterized by massive cellular infiltrates and the disease is associated with abnormalities in the immune system including inappropriate production of antibodies and TC dysfunction. A leaky barrier gains access for luminal antigens in IBD. Innate and adaptive immune cells express a different profile of PRRs. On the other hand microbial antigens from commensals trigger and maintain an inflammatory response. Consequently, mDCs falsely recognize commensals as pathogens, enter a maturation program consisting of an increased expression of PRRs, MHC, and co-stimulatory molecules and finally stop migrating. This process promotes the differentiation of naïve TCs into effector TCs. As a result epithelial cells now also express co-stimulatory molecules which enable them to function as APCs and further contribute to an effector TC response. Several studies provide evidence that the two major forms of IBD in humans are a consequence of dysregulated or excessive T_H1 or T_H2 responses [95-97]. There is considerable evidence that IBD patients have an inappropriate TC response to their own intestinal flora, due to a dysfunction in the primary or secondary mechanisms that normally drive and regulate such responses or based on dysfunction in the intestinal epithelial cell barrier that leads to an inappropriate penetration of microbial agents [98-100].

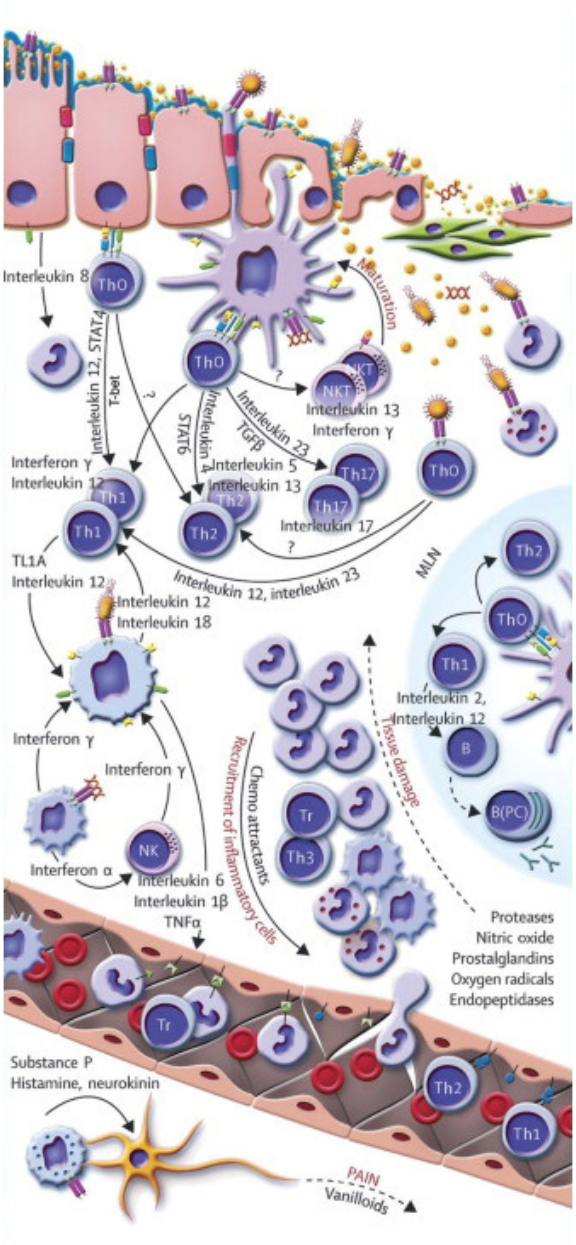

Figure 7: The intestinal immune system in Inflammatory Bowel Disease. In patients suffering from IBD the well controlled balance of the intestinal immune system is destroyed. Antigens gain easy access due to the leaky mucosal tissue. TC – T cell; T_H – T helper cell; T_H0 cells – naïve T cell; T_H, T_H1, T_H2, T_H17 cells – effector TC; T_r, T_H3 – regulatory T cells; B – B cell; B (PC) – plasma cell; NK – natural killer T cell; MLN – mesenteric lymph node. (Adapted from Baumgart et al, 2007 [61]).

Taken together, IBD patients have a failure in maintaining oral tolerance including down-regulation of responses to harmless luminal antigens like commensals or food, while allowing effector cell responses to mucosal pathogens. In active IBD effector TCs predominate over regulatory TCs. Especially in CD, naïve TCs preferably differentiate into IFN-γ and IL-12 producing T_H1 cells whereas in UC these cells differentiate into aberrant IL-5 producing T_H2 cells.

1.6 Probiotics and Inflammatory Bowel Disease

Probiotics are living microorganisms that have a beneficial effect on the host by positively affecting the microbial environment. Typical probiotic candidates are *lactobacilli* or *bifidobacter*, but *E.coli* and other species like yeasts have also been used to study probiotic effects. Probiotic bacteria for humans are preferably of human origin. They have to be safe for the host, genetically stable, and capable of surviving passage through the gastrointestinal tract [101]. Among the effects claimed for probiotics are beneficial immunomodulation, reduction of serum cholesterol, improved lactose digestion and protection against colon cancer [101;102]. It is widely accepted that the intestinal bacterial flora contributes to the pathogenesis of IBD. This could be supported by several experimental and clinical observations in human. The parts of the gut with highest bacterial counts are the sites which are most affected by IBD and antibiotic treatment has lessened disease activity in both patient groups [103]. Commensal bacteria-host interactions are essential in healthy organisms and for immune homeostasis and thus it is not too surprising that disruption of the physiologic bacteria–immune balance may lead to gut inflammation [104;105].

Previously it could be shown that probiotic treatment seems to be effective in patients suffering from IBD [106-108]. The probiotic mixture VSL#3 is effective in both maintenance and prophylactic treatment of pouchitis and also helps to induce remission in patients with UC [109-111]. Further studies could show that the nonpathogenic *Escherichia coli Nissle 1917* is as effective as mesalazine in preventing relapse of UC and *Saccharomyces boulardii* appears useful as maintenance treatment for CD [112]. However, there are also negative sides, as *L rhamnosus GG* and *Lactobacillus johnsonii LA1* are ineffective in preventing postoperative recurrence of CD [113;114]. Additional support for a favorable action of probiotics in gut inflammation comes from animal models, including IL-10- and IL-2– deficient mice, dextran

sodium sulfate- (DSS) and hapten-induced colitis, and HLA-B27 transgenic rats [115-118]. Although clinical results in IBD patients are also encouraging, the data are limited and few studies are placebo-controlled. If probiotics do prove to have beneficial effects in IBD, investigation of the mechanisms may well lead to further advances in treatment.

1.6.1 *Saccharomyces boulardii* in the treatment of Inflammatory Bowel Disease

The probiotic yeast *Saccharomyces boulardii* (*Sb*) in a lyophilized form has demonstrated efficacy in inflammatory and infectious disorders of the gastrointestinal tract in controlled clinical trials [119-124]. The yeast belongs to the group of simple eukaryotic cells, like fungi and algae, and therefore differs from other bacterial probiotics that are prokaryotes. Recent oberservations classified *Sb* genetically within the species of *Saccharomyces cerevisiae* [125-127]. However, it is known that *Sb* differs metabolically as well as physiologically from *S. cerevisiae* [128]. Previous studies evaluated the effect of *Sb* in patients with IBD. It could be demonstrated that the addition of *Sb* to conventional treatment significantly reduced the stool frequency in patients suffering from CD [129]. Furthermore, a beneficial effect in the maintenance of remission in CD has been reported [130]. In patients with left-sided UC, the addition of *Sb* together with mesalamine significantly improved the clinical activity index of these patients [131]. Certainly, little is known about how the probiotic yeast *Sb* unfolds its anti-inflammatory properties in human, especially during IBD.

1.7 Aim

Persistence of IBD is associated with a breakdown of tolerance against the commensal microflora. Previous animal studies have provided insights in the role of mucosal DCs which play a key role in this process. However, the specific function of certain DCs in IBD is still unknown. Thus, primary $CD1c^{+}CD11c^{+}CD14^{-}CD19^{-}$ mDCs or mucosal DCs from IBD patients and healthy controls were compared in this study. Since our group has previously shown that IBD patients with an acute flare-up experience a significant drop in their peripheral mDCs and pDCs populations as well it was important to further characterize the cells from these patients. In the present study experiments were carried out to further specify the localisation and phenotype of human mDCs to gain further insights in possible functional roles of these cells, especially in IBD. Therefore, peripheral blood DCs as well as mucosal DCs were quantified and further analyzed by measuring surface and maturation marker as well as co-stimulatory molecules. Moreover, cytokine secretion of key pro-inflammatory and anti-inflammatory cytokines was investigated to allow a better understanding of the functional role of DCs. Since probiotics become very important as dietary supplements and an effect has been previously shown after probiotic treatment in patients suffering from IBD an additional aim of the current study was to investigate how the probiotic yeast *Saccharomyces boulardii* unfolds its anti-inflammatory properties in human and exceptionally in IBD patients.

2 Material

2.1 Chemicals and supplements

Table 2: Chemicals in alphabetical order.

Name	Company
Ammonium chloride (NH_4Cl)	Sigma Steinheim, Germany
Bacto™ Agar	Difco Laboratories Becton Dickinson Sparks, U.S.A.
Bovine serum albumin (BSA)	Sigma Steinheim, Germany
Carboxyfluorescein-diacetate-succinimidylester (CFSE)	Invitrogen Karlsruhe, Germany
Collagenase NB 8 Broad Range from *Clostridium histolyticum*	Serva Electrophoresis Heidelberg, Germany
DNase I (deoxyribonuclease I) from bovine pancreas Lyophilisate 100 mg	Roche Diagnostics GmbH Mannheim, Germany
DMSO	ICN Biomedicals Inc. Aurora, U.S.A.
Dithienylethene (DTE)	Sigma, Steinheim, Germany
Ethanol	Aaper Alcohol and Chemical Shelbyville. U.S.A.
Fetal calf serum (FCS)	Invitrogen Karlsruhe, Germany
Ficoll-Paque Plus	Amersham Pharmacia Biotech Freiburg, Germany
Fluoromount-G™	Southern Biotech Eching, Germany

Fungizone	Invitrogen Karlsruhe, Germany
Gentamycin	GIBCO™ New York, U.S.A.
Herculase® Enhanced DNA Polymerase	Stratagene La Jolla, U.S.A.
Human serum type AB	Cambrex Charles City, U.S.A.
L-glutamine	GIBCO™ New York, U.S.A.
Lipopolysaccharide, *E. coli* O55:B5	Calbiochem® Darmstadt, Germany
Lipopolysaccharide, from *E. coli serotype* O55:B5 Alexa Fluor 488 conjugate	Molecular Probes Göttingen, Germany
O.C.T.™ Compound	Tissue-Tek® Zoeterwoude, Netherlands
Penicillin-Streptomycin	Biochrom AG Berlin, Germany
Potassium hydrogen carbonate ($KHCO_3$)	Sigma Steinheim, Germany
Propidium Iodide, 50 mg	Calbiochem® Darmstadt, Germany
Pyruvate	GIBCO™ New York, U.S.A.
Recombinant human IL-3	R&D Systems Inc. Minneapolis, U.S.A.
RNase Zap®	Sigma Steinheim, Germany
Sodium azide (NaN_3)	Sigma Steinheim, Germany

Staphylococcal enterotoxin B (SEB)	Sigma
from *Staphylococcus aureus*	Steinheim, Germany
Trypan blue (0.5%)	Sigma
	Steinheim, Germany
YPD-Broth	Sigma
	Steinheim, Germany

2.2 Buffers and solutions

The following buffers and solutions were purchased from commercial suppliers.

Table 3: Buffers purchased from commercial suppliers.

Name	Company
0.5 M EDTA, pH 8.0	Sigma
	Steinheim, Germany
Cytofix/Cytoperm	BD Biosciences
	San Diego, U.S.A.
Dulbecco`s Modified Eagle Medium (DMEM)	GIBCO™
	Grand Island, U.S.A.
FACS Clean Solution	BD Biosciences
	San Diego, U.S.A.
FACS Flow Solution	BD Biosciences
	San Diego, U.S.A.
FACS Rinse Solution	BD Biosciences
	San Diego, U.S.A.
Hanks' Buffered Salt Solution (HBSS)	GIBCO™
	New York, U.S.A.
Perm/Wash Buffer	BD Biosciences
	San Diego, U.S.A.
Phosphate Buffered Saline (PBS)	PAA Laboratories
	Pasching, Austria

RLT-Buffer (Lysis Buffer)	Qiagen Maryland, U.S.A.
RPMI Medium 1640	GIBCO™ Grand Island, U.S.A.

The following buffers and solutions were prepared freshly before use.

Table 4: Freshly prepared buffers and solutions.

Name	Composition
Blocking buffer	PBS 10% FCS
Co-Culture medium	RPMI 0.2% L-glutamine 10% HS type AB 1% Penicillin-Streptomycin
DC culture medium	RPMI 0.2% L-glutamine 10% human serum (HS) type AB 1% Penicillin-Streptomycin 1% Pyruvate
Digest-Mix	Tissue wash buffer 15 mg collagenase 0.2% DNase
Erythrocyte lysis buffer	Aqua dest. 0.1% $KHCO_3$ 0.8% NH_4Cl 0.1 M EDTA at pH 7.5
MACS buffer	PBS 0.4% EDTA 0.5% HS type AB

FACS buffer	PBS 0.05% BSA 0.1% NaN_3
HBSS-DTE	HBSS 5 mM DTE
Tissue wash buffer	HBSS 1% Penicillin-Streptomycin 0.2% Gentamycin 0.4% Fungizone
Washing Buffer	PBS 1% FCS

2.3 Magnetic cell separation reagents

All products for magnetic-activated cell sorting (MACS) were purchased from Miltenyi Biotech, Bergisch Gladbach, Germany.

Table 5: Kits purchased for cell isolation using MACS technology.

Name	Contents
CD1c (BDCA-1)$^+$ Dendritic Cell Isolation Kit	2 ml FcR Blocking Reagent, human: human IgG 2 ml CD19 MicroBeads, human: conjugated to a monoclonal antibody (isotype: mouse IgG1) 2 ml CD1c(BDCA-1)-Biotin antibody, human: monoclonal CD1c(BDCA-1) antibody conjugated to biotin (clone: AD5-8E7; isotype: mouse IgG2a) 2 ml Anti-Biotin MicroBeads: conjugated to monoclonal anti-biotin antibody (isotype: mouse IgG1)
CD4 MultiSort Kit	CD4-Multisort Beads MS Release Reagent MS Stop Reagent
CD45 MicroBeads	2 ml CD45 MicroBeads, human: conjugated to monoclonal anti-human CD45 antibodies (isotype: mouse IgG2a)

CD45RA MicroBeads	2 ml CD45RA MicroBeads, human: conjugated to monoclonal anti-human CD45RA antibodies (isotype: mouse IgG1)

2.4 Antibodies

Table 6: Antibodies, isotype controls and secondary antibodies used for FACS and immunofluorescence staining.

Specificity	Label	Species	Clone	Dilution	Supplier
human CCR7	Alexa 647	rat $IgG_{2a,\kappa}$	3D12	1:20	BD
human CD11c	PE	mouse $IgG_{2b,\kappa}$	sB-ly6	1:10	BD
human CD14	APC	mouse $IgG_{2a,\kappa}$		1:10	BD
	FITC	mouse $IgG_{2b,\kappa}$	TüK4	1:10	Caltag
	PerCp	mouse $IgG_{2a,\kappa}$	MΦP9	1:10	BD
human CD19	Cy5	mouse $IgG_{1,\kappa}$	HIB19	1:10	BD
human CD3	Bio	mouse $IgG2_a$		1:12	Caltag
	PE	mouse $IgG2_a$		1:10	Caltag
human CD4	FITC		S2.5	1:20	Caltag
	PE	mouse IgG_{2a}	SK3	1:10	BD
human CD40	PE	mouse $IgG_{1,\kappa}$	5C3	1:10	BD
human CD45	APC	mouse $IgG_{1,\kappa}$	HI30	1:20	BD
human CD45RA	FITC	mouse $IgG_{2b,\kappa}$	HI100	1:10	BD
human CD80	PE	mouse $IgG_{1,\kappa}$	L307.4	1:10	BD
human CD83	APC	mouse $IgG_{1,\kappa}$		1:10	BD
	FITC	mouse $IgG_{1,\kappa}$	HB15e	1:10	BD
	PE	mouse $IgG_{1,\kappa}$		1:10	BD
human CD86	FITC	mouse $IgG_{1,\kappa}$	2331 (FUN-1)	1:10	BD
	PE	mouse $IgG_{1,\kappa}$		1:10	BD
human anti-CD1c (BDCA-1)	APC	mouse IgG_{2a}	AD5-8E7	1:10	Miltenyi
	FITC	mouse IgG_{2a}	AD5-8E7	1:10	Miltenyi
IFN-γ	PE	mouse IgG_1		1:100	BD
IL-4	PE	mouse IgG_1		1:133	BD

Isotype control	Label	Species	Clone	Dilution	Supplier
IgG_1	FITC	mouse		1:20	Caltag
	PE	mouse		1:20	Caltag
$IgG_{1,\kappa}$	APC	mouse		1:33	BD
	PE	mouse myeloma protein $IgG_{1,\kappa}$	MOPC-21	1:100	BD
IgG_{2a}	Alexa 488	mouse		1:10	Caltag
	APC	mouse		1:10	BD
	APC	rat		1:33	Caltag
	FITC	mouse		1:20	Caltag
	PE	mouse		1:20	Caltag
	PE	rat		1:100	BD
$IgG_{2b,\kappa}$	FITC	mouse		1:10	BD
	PE	mouse		1:10	BD
Secondary Antibody	**Label**	**Species**	**Clone**	**Dilution**	**Supplier**
Streptavidin	Alexa488			1:400	Invitrogen

2.5 Primer

Table 7: Commercial primers for real-time PCR. All primers were purchased from Super Array (Frederick, U.S.A.).

Name	Product number
HPRT	PPH01018B-200
TLR2	PPH01808A-200
TLR4	PPH01795E-200

2.6 Commercial kits

Table 8: Commercial kits.

Kit	Supplier
Advantage® RT-for-PCR Kit	Clontech Mountain View, U.S.A.

BD™ CBA Human Flex Sets	BD Biosciences
(IL-10, IL-6, IL-8, TNF-α)	San Diego, U.S.A.
BD Cytofix/Cytoperm™ 1x Solution	BD Biosciences
	San Diego, U.S.A.
BD™ Cytometric Bead Array (CBA)	BD Biosciences
Human Inflammation Kit	San Diego, U.S.A.
BD Perm/Wash™ 10x Solution	BD Biosciences
	San Diego, U.S.A.
CBA Human Soluble Protein Master Buffer Kit	BD Biosciences
	San Diego, U.S.A.
RNeasy® Mini Kit	Qiagen
	Hilden, Germany

2.7 Consumables

Table 9: General consumables.

Name	Supplier
96 well V-bottom plates	Costar®
	Corning, U.S.A.
96 well Flat-bottom plates	Costar®
	Corning, U.S.A.
Amicon Ultra-15 Centrifugal Filter Units	Millipore
(100 kDa, 50 kDa, 30 kDa, 10 kDa, 3kDa)	Carrigtwohill, Ireland
BD Vacutainer® Heparin Tubes (10 ml)	BD Biosciences
	San Diego, U.S.A.s
Cell strainer (40 µM, 70 µM)	BD Biosciences
	San Diego, U.S.A.
Combitips 0.5 ml, 5 ml, 12.5 ml	Eppendorf
	Hamburg, Germany
Coverslip No. 1 18x18 mm	Germany

Fisherbrand® Microscope Cover Glass	Fisher Scientific Pittsburgh, U.S.A.
Hemacytometer (Neubauer Chamber)	Superior Marienfeld, Germany
Integrid™ Tissue culture dish with 20 mm grid	Falcon, Becton Dickinson Franklin Lakes, U.S.A.
Pap Pen	Dako Glostrup, Denmark
Parafilm®	Pechney Plastic Packaging Menasha, U.S.A.
Petri dishes (100 mm)	Falcon, Becton Dickinson Franklin Lakes, U.S.A.
Pipettes (sterile) 2 ml, 5 ml, 10 ml, 25 ml	BD Falcon®, Becton Dickinson Franklin Lakes, U.S.A.
Polypropylene tubes 15 ml, 50 ml	BD Falcon® Bedford, U.S.A.
Polystyrene Round-Bottom Tube 5 ml	BD Falcon® Bedford, U.S.A.
Reaction tubes 1.5 ml, 2 ml	Eppendorf Hamburg, Germany
SuperFrost® Plus microscope slides	R. Langenbrinck Teningen, Germany
Syringe 5 ml, 10 ml, 20 ml	BD Biosciences Heidelberg, Germany
Tissue culture dish 60x15 mm	BD Falcon®, Becton Dickinson Franklin Lakes, U.S.A.
Tissue culture dish 100x20 mm	BD Falcon®, Becton Dickinson Franklin Lakes, U.S.A.
Weigh dishes (disposable polystyrene)	Fisher Scientific Fair Lawn, U.S.A.

2.8 Material for cell separation

All materials for cell separation were obtained from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany.

LD Columns

LS Columns

MACS® MultiStand

MidiMACS® Separator

MS Columns

OctoMACS® Separator

QuatroMACS® Separator

2.9 Equipment

Table 10: Equipment.

Instrument	Suppliers
ABI PRISM 7500 Fast Lightcycler	Applied Biosystems Foster City, U.S.A.
Centrifuge 5415c	Eppendorf Hamburg, Germany
Centrifuge 5810R	Eppendorf Hamburg, Germany
Centrifuge	neoLab® Heidelberg, Germany
Confocal laser scanning microsope LSM510	Zeiss Jena, sGermany
DU® 640 Spectrophotometer	Beckman Coulter Krefeld, Germany

Flow cytometer: BD FACSCalibur™, BD LSRII™	BD Biosciences Heidelberg, Germany
HLC Blockthermostat BT 1302	HLC Bio Tech Bovenden, Germany
Incubator Hera cell 150	Heraeus Instruments Hanau, Germany
Laboshake	Gerhardt Analytical Systems Königswinter, Germany
LaminAir Flow Bench HBB 2448	Heraeus Instruments Hanau, Germany
Shaker SM-30	Edmund Bühler Germany
Thermomixer compact	Eppendorf Hamburg, Germany
Vortex	Merck® Eurolab Darmstadt, Germany

2.10 Software

Table 11: Software used in this thesis.

Program	Software producer
Acrobat 6.0 Professional	Adobe Systems San Jose, U.S.A.
Adobe Illustrator 11.0.0	Adobe Systems San Jose, U.S.A.
BD Cell Quest™ Pro Version 5.2	BD Biosciences Pharmingen Heidelberg, Germany
FCAP™ Array Software	Softflow New Brighton, U.S.A.
FreeHand® 10	Macromedia San Francisco, U.S.A.

KC-junior Software, version 1.41.4	Bio-Tek Instruments Inc. Winooski, U.S.A.
LSM Image Browser Version 4.0.0.157	Carl Zeiss Jena GmbH Germany
Microsoft Office	Microsoft Redmond, U.S.A.
Photoshop® 7	Adobe Systems San Jose, U.S.A.
Prism® 4	PraphPad Software San Diego, U.S.A.
Reference Manager Version 11	Thomson ISI ResearchSoft U.S.A.
SPSS Version 11.5.1	SPSS Inc. Chicago, U.S.A.

3 Methods

3.1 Overview

For this thesis a special procedure was used to prepare human blood and tissue samples as well as LNs and isolate human mDCs and TCs. Therefore, blood was collected from patients or healthy volunteers and the required cell type was isolated.

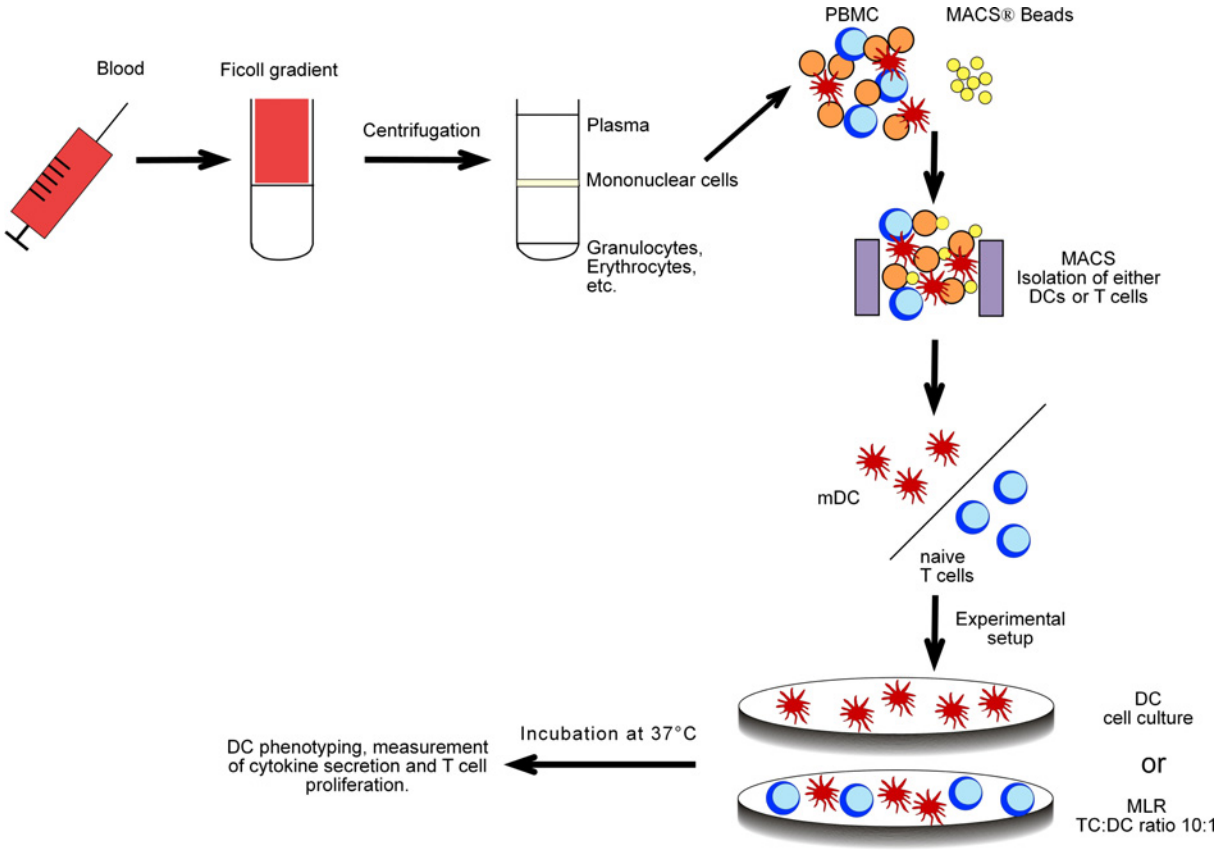

Figure 8: Experimental overview. Blood from patients or healthy volunteers was fractionated via Ficoll density gradient centrifugation and PBMCs were isolated. Consequently mDCs, or TCs were isolated from PBMCs using MACS a magnetic cell separation system. Highly purified DCs were either cultured and phenotyped or further analyzed. Therefore, DCs were co-cultured together with TCs in a mixed lymphocyte reaction (MLR) to further analyze TC proliferation.

3.2 Sample collection and preparation

3.2.1 Human blood and tissue sampling

Peripheral blood as well as mucosal tissue were obtained from patients (n=132) suffering from UC (n=66) or CD (n=66) seen at our Inflammatory Bowel Disease Center. 40 ml blood was drawn and collected into BD Vacutainer® Heparin tubes. As controls either blood from healthy volunteers or leukocyte filters from the blood bank were used (HC=95). For studies with *Sb* further infectious controls (IC, n=15) were analyzed (patients with infectious diarrhea). Controls for mucosa were obtained from patients undergoing surgery for non-inflammatory conditions (Controls=25). CD patients were characterized according to the Montreal Classification [132]. Table 12 summarizes the demographic data.

Table 12: Demographic data of patients and controls. Peripheral blood of patients or healthy controls was used to isolated mDCs which were used in further experiments.

	(n)	%	Mean	Min	Max
Number	132				
Ethnicity					
Caucasian	129	97.7			
Jewish	3	2.3			
Crohn's disease	66	50			
Male	19	14.4			
Female	47	35.6			
Age (years)			40	19	70
Age at diagnosis (years)			30	13	70
Harvey Bradshaw Severity Index Remission			2.8	0	6.5
Harvey Bradshaw Severity Index Flare-Up			11	7	22
Ulcerative colitis	66	50			
Male	30	22.7			
Female	36	27.3			
Age (years)			44	17	85
Age at diagnosis (years)			33	14	60
Modified Truelove Witts Index Remission			4.7	0	9
Modified Truelove Witts Index Flare-Up			11.6	10	16
Infectious controls	15				
Healthy controls	95				

Samples were exclusively analyzed from carefully selected patients off steroids, biologics, immunomodulators and immunosuppressants. The study protocol was approved by Charité's institutional review board. All patients and volunteers gave informed consent to the study.

3.2.2 Scoring of disease activity

Two widely accepted scores, the modified Truelove Witts-Severity Index (MTWSI) for UC and the Harvey Bradshaw Severity Index (HBSI) for CD were used to assess disease activity [133;134]. UC patients who scored ≥10 on the MTWSI and CD patients who scored ≥7 on the HBSI were classified to have active disease (flare-up), scores below these values were classified as patients in remission.

3.2.3 Peripheral blood mononuclear cells

PBMCs can be isolated by Ficoll density-gradient centrifugation. Peripheral blood samples were collected from patients or healthy volunteers. Lymphocytes and monocytes will appear according to their specific density concentrate at the interphase between the upper phase (plasma, thrombocytes) and the lower phase (Ficoll). Erythrocytes and granulocytes of higher density will form a cell pellet.

Briefly, freshly drawn blood from patients or healthy volunteers treated with NH_4-heparin was diluted 1:2 with MACS buffer. Leukocyte removal filter units as used by the Charité blood bank also displayed a source of obtaining control blood. Filter units were wiped with ethanol and all further steps were performed on a clean bench. An arrow on the top of the plastic housing indicated the direction of blood flow during plasma preparation. Using approximately 40 ml of MACS buffer in 20 ml portions and a sterile 20 ml syringe the filter units were flushed in the opposite direction. The extruded blood was collected and the procedure was replicated until the 50 ml falcon tube was completely filled. The diluted blood was then carefully layered over Ficoll Paque (specific density 1.077 g/ml) and was separated by centrifugation at 1000 x g for 20 min at 20°C, without brakes. The interphase containing PBMCs was carefully transferred into a new Falcon tube and washed in 50 ml MACS buffer. Centrifugation of cell suspensions was performed for 6 min at 575 x g at 4°C, unless otherwise stated.

3.2.4 Mucosal mononuclear cells

Lamina propria mononuclear cells (LPMCs) were isolated using a modification of a previously described method [135]. Briefly, surgical specimens were flushed with wash buffer (Tissue wash buffer) and mucus was removed by vigorous shaking in HBSS-DTE. Thereafter, the tissue was cut first longitudinally, then into 5 mm pieces, and incubated in Falcon tubes with 15 ml Tissue wash buffer with constant agitation (200 Mot1/min) at 37°C for 2 hrs. The suspension was then filtered through a sterile metal gauze and finally passed through a 0.25 mm nylon mesh. Erythrocytes were removed using erythrocyte lysis buffer. Cells were washed again, counted and resuspended in MACS buffer.

3.3 Isolation of human blood and mucosal cells

3.3.1 Principle of the magnetic cell sort technology

In order to isolate specific subpopulations of DCs and TCs magnetic cell sort was used. The MACS technology is based on specific labeling of surface molecules with monoclonal antibodies. Therefore, monoclonal antibodies coupled to 50 nm sized paramagnetic MicroBeads which are composed of a biodegradable matrix are used. In brief, cells are magnetically labeled with MicroBeads and separated on a MACS column. The flow-through can be either collected as a negative fraction depleted of the labeled cells or discarded. Finally, labeled cells are eluted. Therefore, the column is removed from the magnetic field and the magnetically retained cells are flushed out as positively selected cells [136].

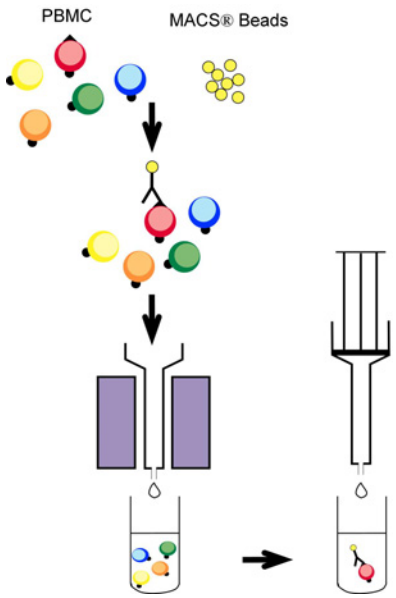

Figure 9: MACS® Technology. 1. Magnetic labeling: cells are magnetically labeled with MACS® MicroBeads in a short incubation step. 2. Magnetic separation: cells are separated on a MACS Column. The flow-through can be collected as negative fraction depleted of the labeled cells. 3. Elution of the labeled cells: the MACS Column is removed from the magnetic field and the magnetically retained cells are flushed out as positively selected cells. (Adapted from Miltenyi Biotec MACS® Cell Separation Strategies.)

3.3.2 Purification of CD45$^+$ mucosal cells

CD45$^+$ cells were isolated using MACS cell separation following a protocol supplied by the manufacturer. In brief, cells were counted and mixed 1:10 with CD45 MicroBeads per 1x10^7 cells. The mixture was incubated for 15 min at 4°C in the dark and washed with MACS buffer. Labeled and unlabeled cells were separated over two LS-column and one MS-column placed in the magnetic field. The purity of the isolated cell population as assessed by FACS was routinely >80%.

3.3.3 Purification of myeloid dendritic cells

mDCs were isolated by magnetic cell separation from PBMCs using CD1c (BDCA-1) antibodies and MicroBeads [137]. In blood the CD1c (BDCA-1) antigen is specifically expressed on DCs, which are CD11c$^+$ CD123$^-$. Additionally a subset of B cells also expresses this antigen. Thus, a depletion of B cells with CD19 MicroBeads was required prior to the enrichment of CD1c (BDCA-1) mDCs. Briefly, CD19$^+$ B cells were directly labeled with

CD19 MicroBeads whereas CD1c (BDCA-1)$^+$ mDCs were labeled with CD1c (BDCA-1) – Biotin. The approach was incubated for 15 min at 4°C in the dark and washed afterwards with MACS buffer. Labeled and non-labeled cells were separated over one LD-column and the B cell-depleted, pre-enriched CD1c (BDCA-1)$^+$ mDC fraction was collected in the column flow-through. The flow-through was washed once with MACS buffer. In a last incubation step pre-enriched CD1c (BDCA-1)$^+$ mDCs were indirect magnetically labeled with Anti-Biotin MicroBeads. After a short incubation step of 15 min at 4°C target cells were eluted from two MS-columns The purity of the isolated CD1c (BDCA-1)$^+$ mDC population was checked with FACS and samples with less than 95% MDC were discarded. The purified mDC population was tested always negative for CD14$^+$ cells.

3.3.4 Isolation of naïve CD4$^+$ T cells

Naïve TCs were isolated from PBMCs using the CD4 MultiSort Kit and CD45RA MicroBeads [137]. In brief, CD4$^+$ cells were positive selected with CD4 MultiSort MicroBeads. Afterwards magnetic particles were enzymatically released, which allows a second magnetic labeling and separation of the cells using CD45RA MicroBeads. CD4$^+$CD45RA$^+$ double positive cells were positively selected from the preselected CD4$^+$ cells. The purity of the isolated naïve TC population was checked with FACS and samples with less than 95% CD4$^+$CD45RA$^+$ double positive TC were discarded.

3.3.5 Characterization of mucosal myeloid dendritic cells

Following enrichment of mucosal CD45+ cells mDCs were identified with CD1c (BDCA-1) antibodies and CD-11c as described in section 3.3.3.

3.4 Cell culture

3.4.1 Preparation of *Saccharomyces boulardii* culture supernatant

Lyophilized *Sb* was provided by Biocodex Laboratories, Montrouge, France. *Sb*S was produced as described previously [138]. In brief, *Sb* was cultured in RPMI 1640 cell culture medium for 24 hrs at 37°C at an initial concentration of 100 mg/ml. To separate the yeast

from the supernatant a 0.2 μm sterile filter was used. Deviant from the published procedure, we did not use a 10-kDa cutoff filter to exclude larger molecules since we were interested in all potential biological active components within the supernatant.

To further analyze the supernatant a serial fractionation strategy was performed using Amicon Ultra-15 Centrifugal Filter Units from Millipore according to the manufactures guidelines. In brief, dissolved yeast supernatant was spun in an Amicon Ultra-15 centrifugal filter device with a 100 kDa nominal molecular weight limit at 4°C. The permeate was spun again in a filter device with a 50 kDa maximum weight limit following centrifugation steps with 30 kDa, 10 kDa, and 3 kDa centrifugal filter devices. The corresponding retentates were collected and kept at -20°C until further use. Retentates were reconstituted with the according amount of RPMI medium before uses.

3.4.2 Culture and stimulation of dendritic cells

$5x10^4$ highly purified mDCs were cultured for 21 hrs in DC culture medium at 37°C and 5% CO_2 in 96-well flat-bottom or 96-well V-bottom plates depending on the experiment which was carried out.

To mimic bacterial microbial activation, mDCs were stimulated with lipopolysaccharide (LPS), a prototypical microbial antigen they may encounter in the gut [28], in the presence or absence of *Sb*S in different dilutions (1:2, 1:8, 1:32) and permeates (<100 kDa, <50 kDa, <30 kDa, <10 kDa, <3 kDa). The particular retentates were added in 1:8 dilutions to cell culture experiments. Ultra pure LPS from *Escherichia coli* 055:B5 was added at a concentration of 100 ng/ml [139].

Finally, mDCs were harvested and stained with the appropriate antibodies to measure the expression of costimulatory molecules and activation marker by FACS. Supernatants were collected for detection of secreted cytokines.

3.4.3 Quantification of intracellular antigen uptake by myeloid dendritic cells

Fluorochrome conjugated LPS was used to study antigen uptake by mDCs [140;141]. Briefly, $2x10^4$ mDCs were incubated for 3, 6, 12, 18 and 24 hrs in DC culture medium supplemented with Alexa Fluor 488 labeled LPS at a final concentration of 2 µg/ml. Unstimulated mDCs served as negative controls. After incubation cells were harvested and each well was rinsed three times with 150 µl PBS. The cell suspension was centrifuged and the cell pellet was washed twice with PBS to remove unincorporated LPS. Cells were finally washed with FACS buffer. Pilot experiments were done to assure that this assay detects intracellular LPS only. Therefore, extracellular fluorescence was quenched with trypan blue before flow cytometry analysis [142]. Since this dye cannot enter intact cells, the fluorescence signal of extracellular bound LPS is being absorbed (quenched). Thus, only cells which possess intracellular LPS are being detected. For trypan blue quenching of fluorescence cells were incubated with a 0.4% trypan blue solution for 1 min at RT. Finally, cells were washed twice to remove the quenching solution. Since no differences in LPS uptake were observed between quenched and unquenched approaches, future experiments were done without quenching.

Finally, mean fluorescence activity (MFI) was measured by FACS at the respective time points following culture. The final MFI was calculated by subtracting the MFI of the negative controls.

3.4.4 Mixed lymphocyte reaction of myeloid dendritic cells and naïve T cells

MLR experiments were performed with freshly isolated mDCs and naïve TCs. The MLR is a special technique based on the measurement of histocompatibility at the HL-A locus. Originally this experiment was developed as a model for the graft-versus-host disease. Peripheral blood lymphocytes from two individuals are mixed together in tissue culture for several days. Lymphocytes from incompatible individuals will stimulate each other to proliferate significantly, measured by CFSE uptake, whereas those from compatible individuals will not.

For MLR experiments $1x10^4$ highly purified mDCs were preincubated with LPS (12.5 ng/ml) in the presence or absence of *Sb*S in different dilutions (1:2, 1:8, 1:32) and permeates (<100 kDa, <50 kDa, <30 kDa, <10 kDa, <3 kDa) for 3 hours at 37°C and 5% CO_2 in Co-culture medium. The particular retentates were added in 1:8 dilutions to cell culture experiments. Afterwards $1x10^5$ isolated naïve, allogenic and CFSE labeled TCs from healthy volunteers were added and co-cultured for 5 days at 37°C and 5% CO_2 in 96-well V-bottom plates.

A total of 1 x 10^5 TCs were cultured with DCs at a 10:1 ratio in 96-well V-bottom plates. To rule out TC unresponsiveness in MLR experiments staphylococcal enterotoxin B (SEB) was added to some control experiments with naïve TCs at a concentration of 6.7 µg/ml.

3.5 FACS

3.5.1 Principles of flow cytometry

A flow cytometer is an instrument that illuminates cells as they flow individually in front of a light source and then detects and correlates the signals from those cells that result from the illumination. The principle of a flow cytometer is based on simultaneous measurement of multiple physical and chemical properties while single cells pass through the measuring apparatus in a fluid. In this thesis, data were collected on a FACSCalibur and the following analysis was preformed using the CellQuest software.

Flow cytometers incorporate a light source, associated illumination optics (lenses), light collection optics (filters) and photodetectors or photomultipliers. The FACSCalibur used in this thesis was equipped with a 488 nM argon laser and 635 nM red diode laser as a light source. With the fluorescence activated cell sort (FACS) one can quantify surface receptors as well as intracellular proteins like cytokines. The analysis based on the specific interaction of antigen with monoclonal antibodies, which are conjugated to fluorescent dyes. A single file flow of cells is produced by introducing the sample fluid containing a single cell suspension through a narrow injector into a wider capillary through which a cell-free sheath is flowing under pressure. The cytometer is adjusted that only one cell at the time can pass the intersecting laser beams. Therefore, the diameter of the stream is gradually reduced behind the injection point. The cells are measured at specific points which are termed the observation points. At these points the laser beams are brought into focus which otherwise orthogonally

intersect the stream in the capillary. During the analysis the suspended cells are hydrodynamical focused and pass a focused laser beam of appropriate wavelength. Thereby the electrons of the dye are excited by the monochromatic laser beam and reach a higher energy level. After passing the laser beam, the electrons loose their energy (in form of photons) and emmit light with longer wavelength, which then can be detected by a detector. The amount of emmitted photons is related to the amount of bound antibodies per cell. Additional information is given through the scatter signals of the laser itself. The forward scatter signal (FSC) is related to cell size, whereas the side scatter signal (SSC) says something about the granularity of the cytoplasm or the surface roughness of a cell. A simultaneous measurement of up to four different fluorescent dyes is possible during the usage of the FACSCalibur, because of similar extinction properties and different emmission spectras.

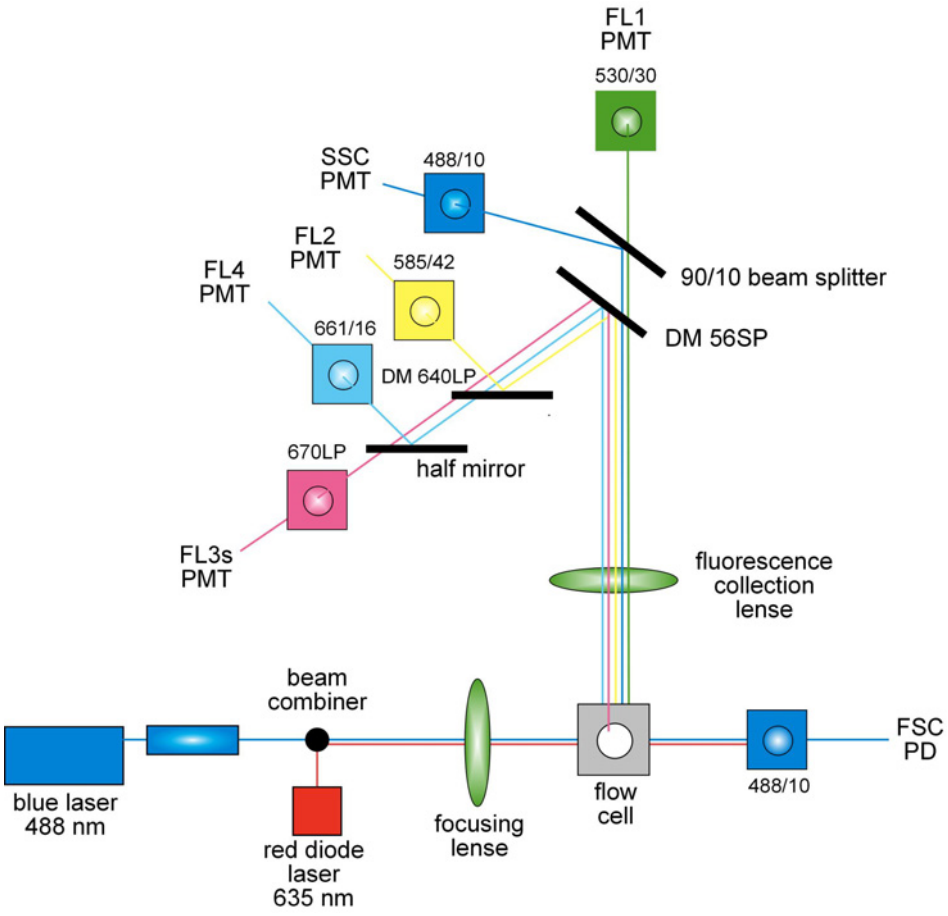

Figure 10: Optics of a FACSCalibur. Optics of a multi-laser flow cytometer with the two lasers focusing at different locations along the sample stream to prevent cross-contamination from the other beam. FSC – forward scatter, SSC – side scatter, FL – fluorescent light, LP – long pass, SP – short pass, DM – dichroic mirror, PD – photo diode, PMT – photomultiplier. (Adapted from BD Immunocytometry Systems FACSCalibur System User's Guide.)

In the work described herein, cells were directly labeled with fluorochrome coupled antibodies of different specificities. The fluorochromes listed in table 13 were used in variable combination in this thesis. For detection of the emitted fluorescence optical filters are used. They absorb or reflect light above or below a particular wavelength, termed the passband. Depending on the fluorochromes being used, these optical components limit the spectral range of the emitted light after passage through the fluorescence collection lens. However, even optimal filter cannot avoid a certain degree of overlap between, the emission spectra of two fluorochromes. Therefore, an electronic compensation is necessary before analyzing the samples. While the FSC and SSC detectors are photoelectric photodiodes, photomultiplier tubes are used to detect fluorescence as they contain an internal amplification mechanism.

Table 13: FACSCalibur detectors and filter configuration. The FACSCalibur has 2 laser lines (488 nm blue and 635 nm red) and 4 colour detectors. FL – fluorescent light, LP – long pass, BP– band pass optical filters, PMT – photomultiplier, * APC and PE-Cy5 may be used together on instruments with cross-beam compensation, Ex – Extinction, Em - Emission.

PMT	Filter	Colour	Fluorochrome	Ex-Max [nm]	Em-Max [nm]
FL1	530/30 BP	Green	Alexa Fluor 488	495	519
	(515 – 545 nm)		FITC	494	519
			CFSE		
FL2	585/42 BP	Yellow / Orange	PE	496, 546	578
	(564 – 606 nm)		PI		
FL3	670 LP	Far Red	PE-Cy5*	496, 546	615
	(>670 nm)		PerCP	482	678
			PI		
FL4	661/16 BP	Red	Alexa Fluor 647	650	688
	(653 – 669 nm)		APC*	650	660

Lymphocytes exhibit characteristic FSC and SSC properties when analyzed by FACS. Thus they can easily be discriminated from other cells like macrophages, granulocytes, or erythrocytes. Consequently, it is possible to identify living lymphocytes and specify a gate to further analyse the cells alone, known as the lymphocyte-live gate. To identify distinct lymphocyte subpopulations, use is made of discriminating phenotypic characteristics revealed by particular fluorochrome coupled antibodies bound to the cells [143]. Therefore all acquired

cells are first analyzed for two FL parameters. Afterwards, cells with a distinctive pattern can be electronically selected in a process called gating and further analyzed for other parameters.

3.5.2 Staining procedure

Three or four-color flow cytometric analysis was used to identify and enumerate mDCs and naïve TCs [144]. Briefly, cells were directly stained for 15 min at 4°C in the dark with the appropriate amount of antibodies against specific surface molecules which were conjugated with one of the following fluorochromes shown in Table 13. Unbound antibody was removed by washing the cells with FACS buffer. The suspension was centrifuged and the supernatant was discarded. The cells were finally resuspended in 500 µl FACS buffer and analyzed immediately. Prior to running the samples, propidium iodide (PI) at a final concentration of 0.1 mg/ml was added to label dead cells in most of the measurements.

3.5.3 Acquisition and analysis

Data was collected and analyzed using Cell Quest™ software.

Combination of many fluorochromes requires careful compensation in order to minimize spectral overlap into neighbouring channels. In order to set up a compensation matrix, single stained samples were prepared with each of the fluorochromes used in the respective experiment. Unstained cells as well as single stained compensation samples were run prior to the experiment and compensation was performed.

Advanced gating strategies were used to exclude dead, i.e. PI positive, cells and debris. A major limitation in immunofluorescence measurements is the nonspecific labeling of damaged and nonviable cells by some antibodies and the inability to distinguish nonspecific from specific antibody labeling. Dead or damaged cells can represent a significant source of error in FACS analysis of viable cells due to nonspecific uptake of labeled antibody probes, increased cellular autofluorescence and altered cell-surface antigen expression. Light scatter has been used to discern nonviable from viable cells in homogeneous populations [143]. Another method of identifying damaged or nonviable cells has been the use of red-emitting fluorochromes which bind to nucleic acids by intercalation, i.e. PI. When used at low concentrations, these highly sensitive dyes penetrate intact viable cells slowly, but rapidly

enter damaged cells where they bind to nucleic acids and fluorescence bright red. Both methods light scatter (FSC / SCC) and PI were combined to exclude unspecific staining and dead cells [143].

If not indicated otherwise, plots shown in this work are representatives for at least 5 experiments.

3.5.4 CFSE staining of T cells

In order to follow proliferation of TCs in co-culture experiments *in vitro* freshly isolated TCs were labeled with the amin-reactive cell tracker dye 5-carboxyfluorescein-diacetate-succinimidylester (CFSE). CFSE is a fluorescent dye that can be used to measure cell proliferation using flow cytometry. This membrane-penetrating dye is transported into the cell during incubation with mononuclear cells and binds covalently to amino groups of cellular proteins without adversely affecting cellular function. The cleavage of acetate groups by intracellular esterases yields in a highly fluorescent membrane-impermeable product and results in a uniformly labeled cell population. During cell division the fluorescent dye is gradually lost and thereby equally distributed to the daughter cells. This mechanism allows the identification and enumeration of proliferating cells [145;146]. Analysis of cell division can be determined through its intensity when measured by flow cytometry. With each cell division, the fluorescent intensity per cell is halved, thus providing a readout of the mitotic activity within a specific population of cells.

For labeling TCs were washed twice in PBS. Finally, an equal amount of CFSE solution was added to the TC (5 µM CFSE) suspension and incubated for 4 min at RT at the dark. CFSE labeling decreased at each cellular division, but as DCs do not divide, they remain highly positive for CFSE and could be eliminated with special gating strategies [147]. The cells were washed with at least 10-fold excess volume of co-culture medium terminated the reaction. Labeled cells were subsequently activated *in vitro* by procedures described above. Analysis of proliferation was done 5 days after labeling by flow cytometry.

3.5.5 Intracellular cytokine staining

For intracellular cytokine staining, TCs were co-cultured with mDCs as described above (see 3.4.4) for the indicated period of time. 5 hrs before the staining cells were restimulated with 1 µg/ml Ionomycin, 10 ng/ml Phorbol Myristate Acetate (PMA), and 1:10 of the Golgi Stop Solutions. After the indicated time of culture, the cells were harvested, washed once in FACS buffer and fixed in 250 µl Cytofix/Cytoperm Buffer for 20 min at 4°C. Cells were washed twice with 1 ml of BD Perm/Wash buffer. Finally, intracellular staining was performed in a total volume of 100 µl for 30 min at 4°C. For intracellular IFN-γ or IL-4 staining PE- and APC-conjugated monoclonal rat anti-mouse antibodies and their isotype control antibodies were used, respectively. Unbound antibody was removed by washing twice with 1 ml of Perm/Wash Buffer, cells were resuspended in FACS buffer and analyzed by flow cytometry. As a control an unstained approach was prepared.

3.5.6 Cytometric bead array analysis of cytokine secretion by dendritic cells

Cytokine secretion in culture supernatants was measured by cytometric bead array (CBA) analysis according to manufacturer's guidelines. CBA a basic “sandwich” multiplexed bead assay, is a series of spectrally discrete particles which can be used to capture and quantitate soluble analytes. The analyte is measured by detection of a fluorescence-based emission and flow cytometric analysis. CBA assays are multiplexed such that numerous substances are measured simultaneously in a single well. The CBA assay consists of a mixture of several types of beads which were randomly picked and with a uniform size but containing different fluorescence intensities of a red-emitting dye. A different capture antibody against one of the analyzed cytokines is covalently coupled to each type of bead. Cytokines bound to these antibodies are finally detected by the use of antibodies labeled with PE. The fluorescence intensity measured with PE is proportional to the cytokine concentration in the sample and can be quantified from a calibration curve.

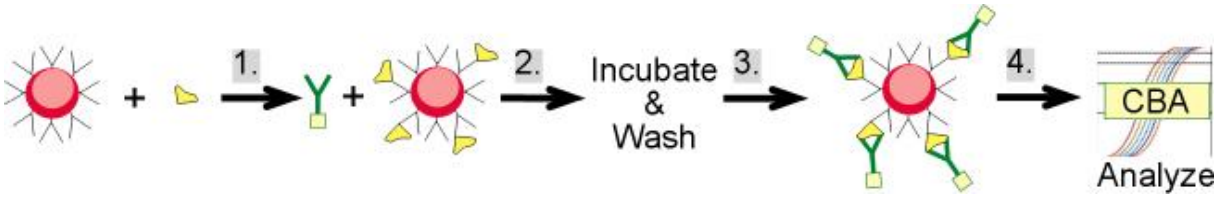

Figure 11: Principle of Cytometric Bead Array. A Cytometric Bead Array (CBA) commonly referred to as a multiplexed bead assay. Simultaneous detection of multiple cytokines is the result of a series of spectrally discrete particles that can be used to capture and quantitate soluble analytes. The capture beads (1.), PE-conjugated detection antibodies (2.), and recombinant standards or test samples are incubated together to form sandwich complexes (3.). Following acquisition of sample data using the flow cytometer, the sample results are generated in graphical and tabular format using the BD™ CBA Analysis Software (4.). (Adapted from BD Biosciences.)

Briefly, bead populations with distinct fluorescence intensities were incubated with recombinant standards or test samples for 1 hr at RT. 50 µl PE-conjugated detection antibodies were added to each approach and incubated for 2 hrs at RT in the dark. Samples were washed with Wash Buffer at 200 x g for 5 min. The supernatant was discarded carefully and the analysis was performed in 300 µl Wash Buffer. With this assay the production of TNF-α, IL-6, IL-8 and IL-10 by mDCs was measured. After acquisition of sample data using FACS, the cytokine concentrations were calculated using the proprietary FCAP™ analysis software [138;148].

3.6 RNA Isolation and cDNA synthesis

In order to perform RT-PCR experiments to look for TLR expression in cultured human mDCs total RNA was prepared from cell pellets by using the RNeasy Mini Kit from Qiagen, following manufacturer's instructions. In brief, freshly isolated or harvested cells from culture experiments were lysed in 350 µl RLT-buffer + 3.5 µl β-Mercaptoethanol and total RNA was extracted.

cDNA was synthesized using the Advantage™ RT-for-PCR Kit from Clontech. Briefly, 12.5 µl of the isolated RNA were used for cDNA synthesis. The procedure was carried out on ice. 1 µl of the random hexamer primer was added to each approach. To remove secondary structure, the RNA extracts were heated for 2 min at 70°C in the thermomixer. For cDNA synthesis, 6.5 µl of RT reaction mixture (Table 14) were added.

Table 14: cDNA Synthesis.

Reagent	Volume [µl]
5x reaction buffer	4.0
dNTP mix (10 mM each)	1.0
Recombinant RNase inhibitor	0.5
MMLV reverse transcriptase	1.0
Total volume	**6.5**

The reaction mixtures were incubated at 42°C for 1 hr. Afterwards the cDNA synthesis reaction was stopped and any DNase activity was destroyed by heating the reaction mixtures at 95°C for 5 min. 80µl of DEPC-treated water were added to each sample to make up a total volume of 100 µl before performing real-time PCR.

3.7 Quantitative real-time RT-PCR

The polymerase chain reaction (PCR) is a method for generating defined DNA sequences. Therefore a heat stable DNA polymerase enzymatically amplifies a DNA target sequence. The reaction mixture includes a suitable amount of template DNA, a specific oligonucleotide primer pair that flanks the target DNA sequence and hybridizes to opposite DNA strands, nucleotides and a special buffer system. Repetitive series of cycles, which include template denaturation, primer annealing and elongation result in an exponential accumulation of the specific DNA sequence. A comparatively new PCR method is the real-time PCR. It allows to actually view the increase in the amount of DNA as it is amplified. Several different types of real-time PCR are available at this time, TaqMan® real-time PCR, as well molecular beacon and SYBR® Green.

Expression of TLR2 and TLR4 was analyzed by quantitative real-time PCR using specific primers and the SYBR Green dye as the fluorescent reporter. PCR reactions were performed with 1µg of cDNA using an ABI PRISM 7500 Fast Lightcycler. Relative quantification was assessed by using the formula $2^{-\Delta CT}$ and by normalizing the amount of the target gene to the housekeeping gene HPRT. The real-time PCR program consisted of one cycle of 95°C for 15 min, and then 40 cycles of 95°C for 15 sec, 60°C for 1 min. The dissociation was carried out at 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec.

3.8 Statistical Analysis

The results obtained in this study were either represented as single values or are expressed as mean ± standard error of the mean (SEM). Comparisons are by a two tailed Mann-Whitney U test with statistical significance accepted for $p < 0.05$. For evaluation of cytokine secretion, one way analyses of variance (ANOVA) with Tukey's Multiple Comparison Test as post-test were used for intergroup comparison.

4 Results

4.1 High purity of isolated human myeloid dendritic cells from peripheral blood

For the present study highly pure DCs were acquired from human peripheral blood. Previous work from our group showed that DCs could be isolated from peripheral blood of healthy volunteers and IBD patients [144]. For this thesis mDCs as well as naïve TCs could be isolated with a purity of at least 95% (Figure 12), respectively.

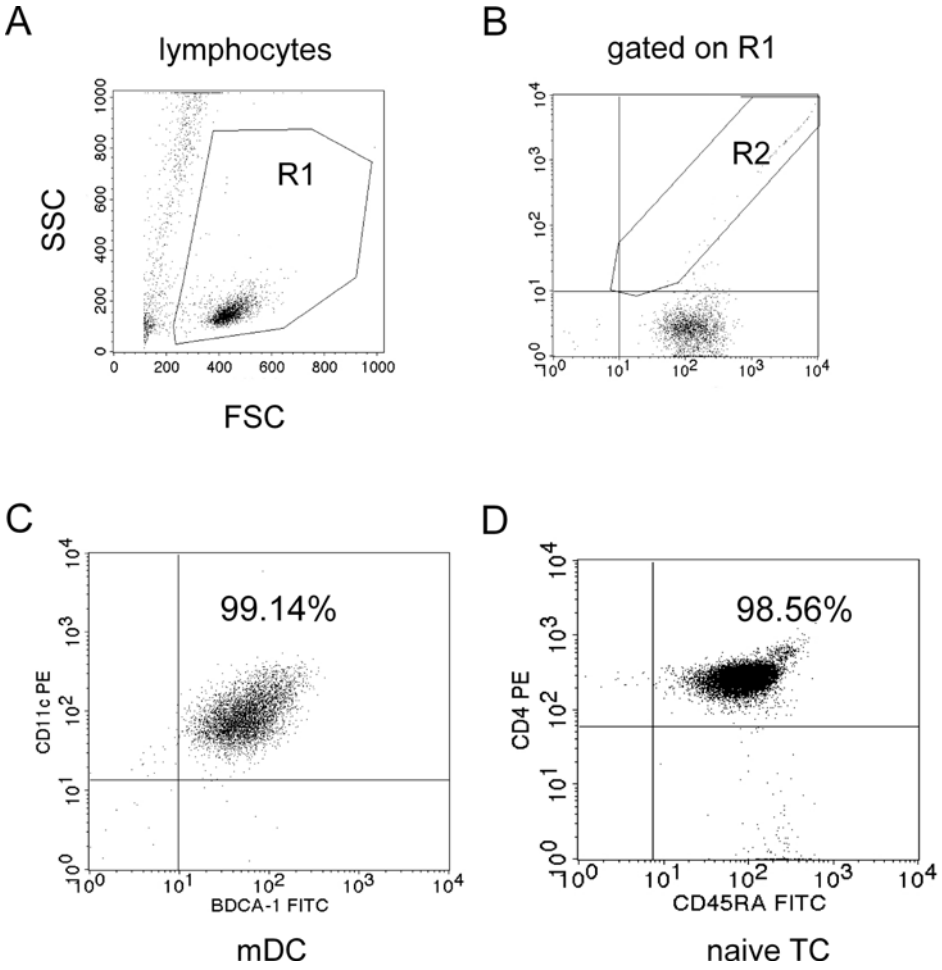

Figure 12: Isolation of human myeloid dendritic cells and T cells from peripheral blood. mDCs and naïve TCs were isolated from human peripheral blood and stained with fluorochrome-labeled antibodies. (A) Representative dot plot shows FCS vs. SSC of all acquired events. A region (R1) was drawn on lymphocytes according to their known scatter properties excluding debris from further analysis. (B) Dot plot represents PI-positive cells. A new region (R2) was drawn on all PI-positive lymphocytes within R1. All analyzed cell types were gated on R1 without the PI-positive cells within region R2. (C) Flow cytometry analysis of isolated mDCs stained for CD11c and CD1c (BDCA-1). All isolated mDCs were negative for CD14 and CD19 (staining not shown). (D) The right panel shows naïve TCs which were stained for CD4 and CD45RA, respectively. All dot plots show representatives for the particular isolated cell type. SSC – Side Scatter Signal, FSC – Forward Scatter Signal.

4.2 Phenotype and development of human dendritic cells in the periphery

This part of the project was initiated in order to analyze and further characterize human DCs from healthy volunteers in comparison with DCs from IBD patients. Of particular importance were mDC subpopulations which were characterised by the expression of specific activation marker and co-stimulatory molecules.

4.2.1 More myeloid dendritic cells from IBD patients display an activated phenotype than controls

It is hypothesized that mDCs from IBD patients may induce and/or perpetuate inflammation. Therefore, in the thesis presented here, the marker profile of human blood mDCs and how they regulate their phenotype in response to LPS, a prototypical microbial antigen they may encounter in the gut was investigated [28]. First, the expression of CD40 and CD80, two co-stimulatory molecules which are known to be up-regulated on mature and activated human DCs and critical for the activation of naïve TCs, was studied [138;149-151].

In UC, the fraction of CD40 expressing mDCs was significantly higher in cultured mDCs compared with controls (Figure 13B left panel). LPS stimulation further increased the number of CD40 expressing mDCs during RM and FU compared with controls (Figure 13C left panel). A similar pattern was observed in patients suffering from CD. Here the fraction of CD40 expressing mDCs was significantly higher in cultured mDCs in comparison to healthy controls (Figure 13B right panel). Additional stimulation with LPS further increased this expression as well (Figure 13C right panel). In contrast, no significant difference between CD40 expressing mDCs among IBD patients and healthy controls could be observed in freshly isolated mDCs (Figure 13A left and right panel). mDCs from healthy volunteers as well as from patients suffering from UC or CD express virtually no CD40.

Overall, CD40 regulation was comparable between UC and CD patients. No differences between CD40 expressing mDCs during RM and acute FU in both types of disease could be observed.

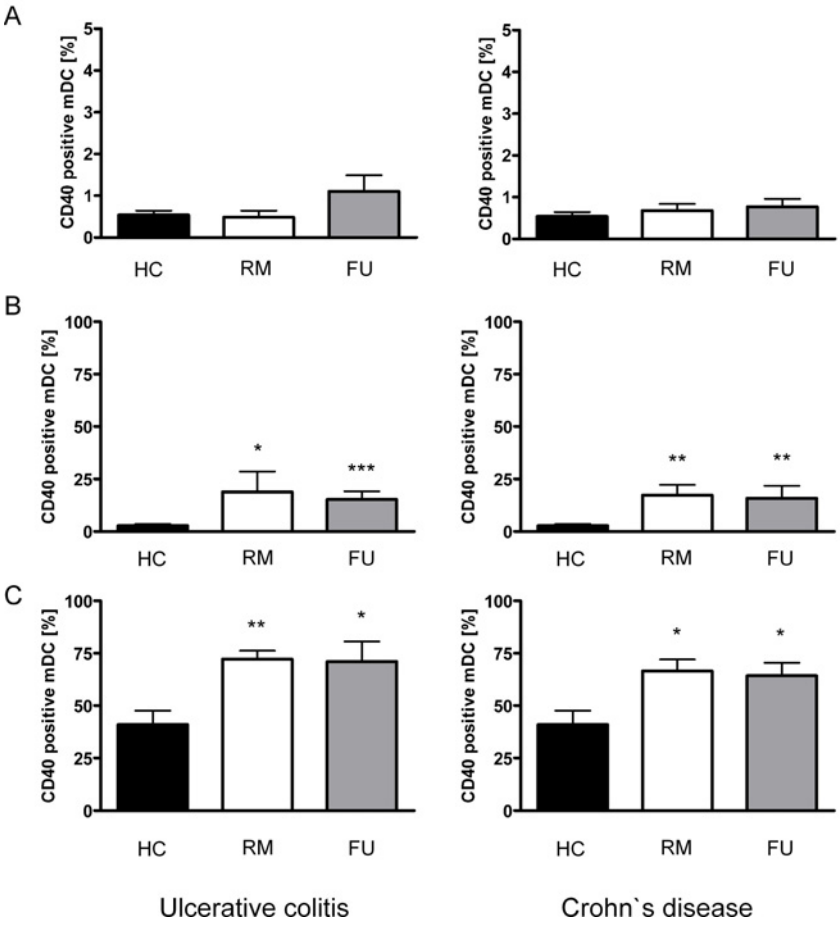

Figure 13: CD40 Expression. (B) More cultured and (C) LPS stimulated, but not (A) freshly isolated mDCs, express CD40 in IBD compared with healthy controls. Bar graphs summarize data from 10 healthy controls and 6 patients in each category. HC – Healthy Control, RM – Remission, FU – Flare-up. Asteriks denote statistical significance $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

Furthermore, the expression pattern of CD80 was studied. Freshly isolated mDCs from healthy controls as well as from IBD patients showed virtually no CD80 expression (Figure 14A). No significant differences could be observed for freshly isolated cells.

In patients suffering from UC, the fraction of CD80 expressing mDCs was slightly higher in cultured mDCs compared with controls (Figure 14B). Patients come down with CD showed likewise a slightly higher fraction of CD80 expressing mDCs in comparison to healthy controls. LPS stimulation further increased the number of CD80 expressing mDCs for all approaches whereas in patients during an acute FU the number of CD80 expressing mDCs was significantly higher compared with controls (Figure 14C). However, in CD the number of CD80 expressing mDCs after stimulation with LPS was also significantly increased during

RM when compared with controls. This effect could not be observed in patients with UC. Here, only patients during an acute FU showed a significant increase in the number of CD80 expressing mDCs. No differences were observed between patients reside in RM and patients during an acute FU could be observed for both types of disease.

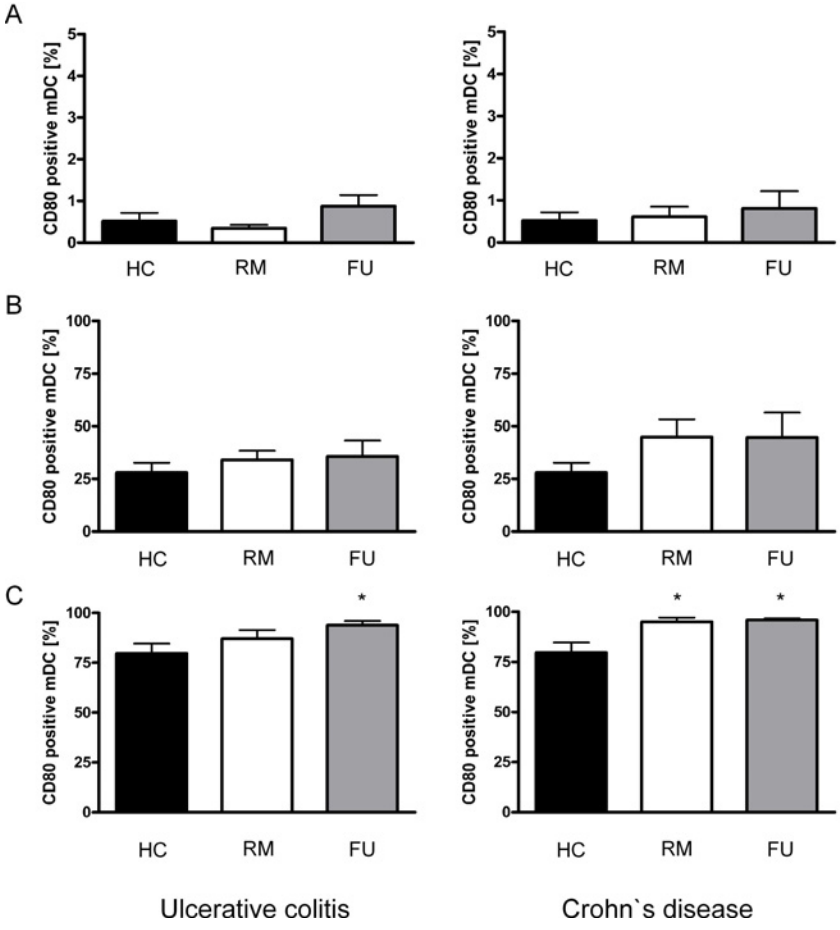

Figure 14: CD80 Expression. (B) More cultured and (C) LPS stimulated, but not (A) freshly isolated mDCs, express CD80 in IBD compared with healthy controls. Bar graphs summarize data from 7 healthy controls and 6 patients in each category. HC – Healthy Control, RM – Remission, FU – Flare-up. Asteriks denote statistical significance $p < 0.05$ = *.

4.2.2 Myeloid dendritic cells from IBD patients secrete more inflammatory cytokines upon LPS stimulation than controls

Based on the previous observation in which an activated and more mature phenotype of cultured and LPS stimulated mDCs in IBD patients was identified, the following experiments addressed the production of key IBD cytokines known to be produced by DCs such as TNF-α,

IL-6, IL-8 and IL-10. Cytokines play an important role in IBD that determine TC differentiation of T_H1, T_H2, regulatory TCs as well as T_H17. Since there is evidence for a disturbed balance between pro-inflammatory and anti-inflammatory cytokines in patients suffering from IBD the secretion of major cytokines was investigated [152].

First the secretion of IL-6, a major acute phase reactant, was studied (Figure 15). IL-6 is often induced together with other pro-inflammatory cytokines IL-1 and TNF-α. Cultured mDCs secrete very low levels of IL-6. However, mDCs from UC and CD patients secreted approximately 3 times more IL-8 than in controls. The addition of LPS causes an increase in IL-6 secretion (Figure 15B). In CD patients no significant differences could be observed when compared to controls. On the other hand, patients suffering from UC secrete significantly more IL-6 during RM compared to healthy controls. Remarkable was the observation that flaring UC patients secrete significantly less IL-6 than in RM.

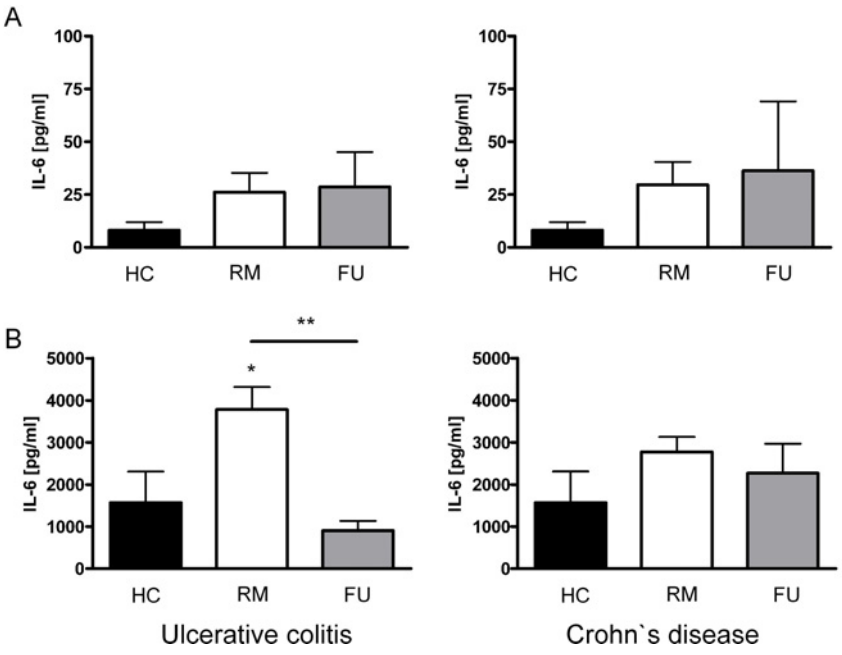

Figure 15: IL-6 secretion of human myeloid dendritic cells. (A) Cultured mDCs from healthy controls and IBD patients show a low secretion of IL-6. (B) LPS stimulated mDCs from CD patients secrete slightly more IL-6 than healthy controls. IL-6 secretion of CD patients is significantly increased in RM and decreases during FU. Bar graphs summarize data from at least 6 patients and 8 controls within each category. UC – Ulcerative Colitis, CD – Crohn's Disease, HC – Healthy Control, RM – Remission, FU – Flare-up. Asteriks denote statistical significance $p < 0.05$ = *, $p < 0.01$ = **.

CXCL8 is commonly known as IL-8 which is a powerful neutrophil chemoattractant and activator [153]. Therefore, at the site of inflammation a role for IL-8 and its regulatory cytokines IL-1 and TNF-α in the recruitment and transmigration of neutrophils in IBD has been suggested [152;154]. Here, cultured mDCs from UC and CD patients secreted approximately 1.5 times more IL-8 than healthy controls. Overall, small amounts with no significant differences between all groups and substantially less compared with stimulated cells were secreted (Figure 16A). LPS stimulated mDCs from patients with UC and CD secreted significantly more IL-8, than controls (Figure 16B).

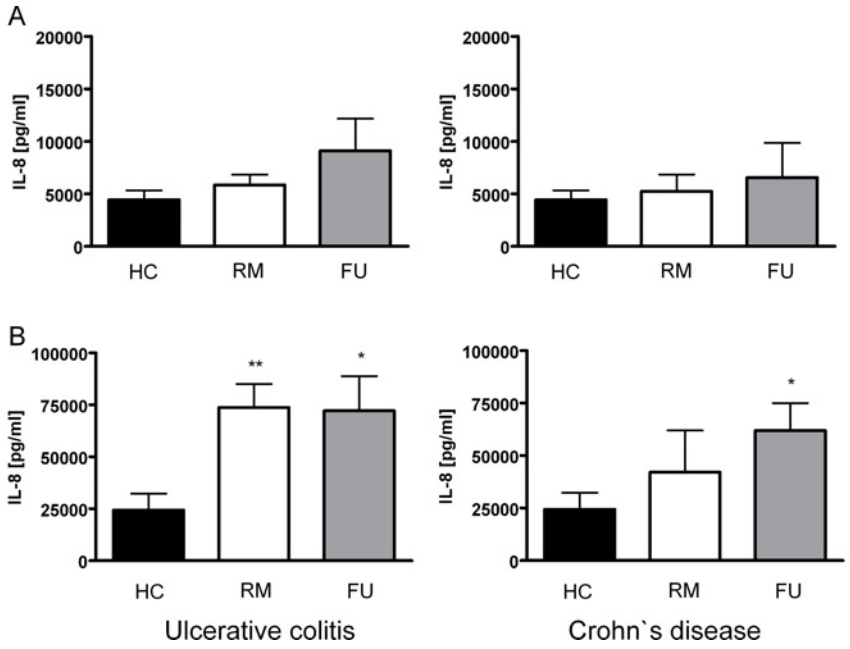

Figure 16: IL-8 secretion of human mDCs. (A) Cultured mDCs secrete high levels of IL-8 but without any differences between patients and healthy volunteers. (B) LPS stimulated mDCs from IBD patients secrete significantly more IL-8. Bar graphs summarize data from at least 6 patients and 8 controls within each category. UC – Ulcerative Colitis, CD – Crohn's Disease, HC – Healthy Control, RM – Remission, FU – Flare-up. Asteriks denote statistical significance $p < 0.05$ = *, $p < 0.01$ = **.

Since DCs are known to have a dual role in inflammation and may also help control inflammation independent of regulatory TCs, IL-10 secretion was quantified as well [23;155]. Anti-inflammatory IL-10 is able to attenuate a mucosal inflammation due to its ability to inhibit both antigen presentation as well as the release of pro-inflammatory cytokines. Unstimulated, cultured mDCs do not secrete any significant amounts of IL-10 (Figure 17A). In turn, LPS stimulated mDCs from patients suffering from UC and CD secrete significantly

more IL-10, than controls. Of note, IL-10 secretion by LPS stimulated mDCs from flaring UC patients was lower than in RM. However, this trend was not noticed in patients with CD (Figure 17B).

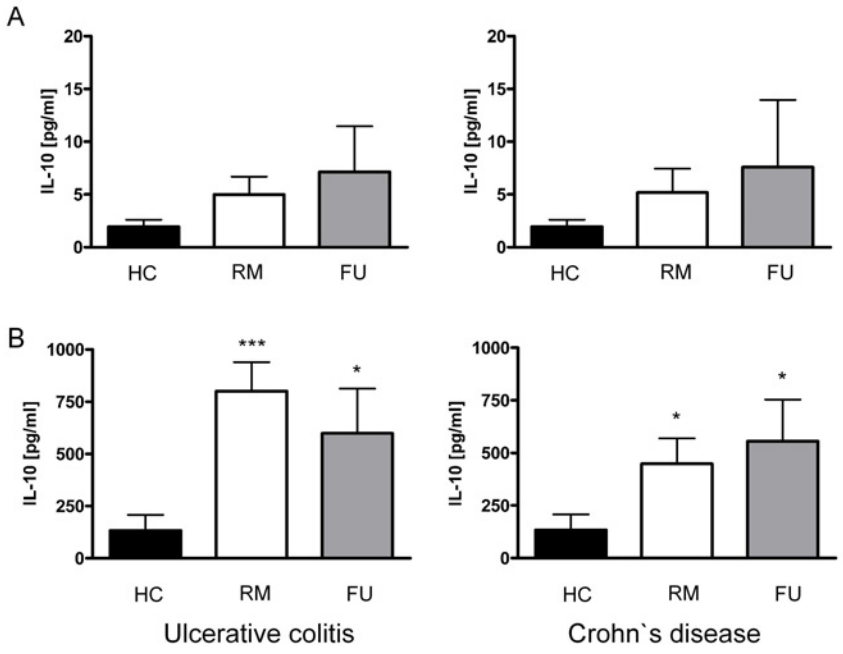

Figure 17: IL-10 secretion of human myeloid dendritic cells. Cultured mDCs show a very low secretion of IL-10. (A) IL-10 secretion of human mDCs from healthy volunteers and IBS patients. (B) LPS stimulated mDCs from IBD patients secrete significantly more IL-10 than healthy controls. Bar graphs summarize data from at least 6 patients and 8 controls within each category. UC – Ulcerative Colitis, CD – Crohn's Disease, HC – Healthy Control, RM – Remission, FU – Flare-up. Asteriks denote statistical significance $p < 0.05$ = *, $p < 0.001$ = ***.

Finally, the secretion of TNF-α was studied which is an important mediator of inflammation. Cultured mDCs from UC patients secreted equivalent TNF-α when compared with healthy controls (Figure 18A). However, mDCs from CD patients secrete up to 1.8 times more TNF-α compared with healthy controls, but overall small amounts with no significant differences between all groups and substantially less than stimulated cells. LPS stimulation directed to an increase in the secretion of TNF-α as well (Figure 18B). LPS stimulated mDCs from UC patients and CD secreted significantly more TNF-α, than controls. Interestingly, the TNF-α secretion in flaring patients was lower, than in RM maybe due to exhaustion.

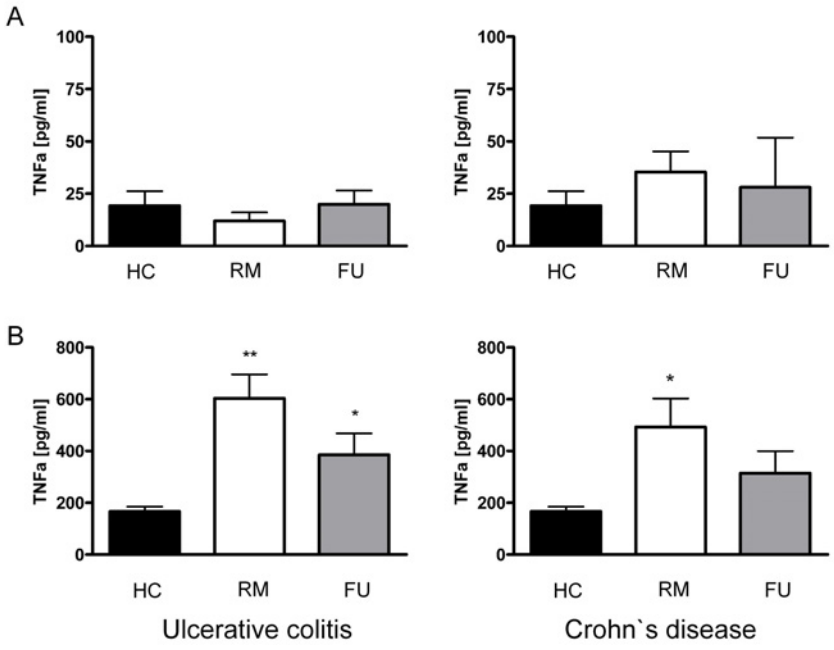

Figure 18: TNF-α secretion of human myeloid dendritic cells. (A) Cultured mDCs from healthy controls and IBD patients show low secretion of TNF-α. (B) Further stimulation with LPS lead to an increase in the secretion of TNF-α. Stimulated mDCs from IBD patients secrete significantly more TNF-α than healthy controls. Bar graphs summarize data from at least 6 patients and 8 controls within each category. UC – Ulcerative Colitis, CD – Crohn's Disease, HC – Healthy Control, RM – Remission, FU – Flare-up. Asteriks denote statistical significance $p < 0.05$ = *, $p < 0.01$ = **.

Taken together, mDCs from healthy controls as well as from IBD patients showed an increase in the cytokine secretion after stimulation with LPS. Secretion of pro-inflammatory IL-8 showed the highest value in all groups compared with the other cytokines that were studied. These results further support the observed phenotypic differences discussed above. It could be shown that mDCs from IBD patients secrete greater amounts of cytokines which were tested in this thesis than cells from healthy volunteers.

4.2.3 Myeloid dendritic cells effectively stimulate naïve T cells in an alloreaction and lead to a differentiation into effector T cells

In an allogenic MLR mDCs and naïve TCs which were isolated from healthy donors were co-cultured for 5 days. Afterwards the proliferation was measured and an intracellular staining for IL-4 and IFN-γ was performed (Figure 19).

Naïve TCs showed some autoproliferation in the presence (0.95 ± 0.12%) and absence (0.85 ± 0.13%) of LPS. The MLR of mDCs and naïve TCs induced a robust TC proliferation (49.36 ± 3.3%). Further addition of LPS to the alloreaction did not change the potential to proliferate (54.05 ± 1.8%). To rule-out TC anergy in this set of experiments SEB was used as a positive control. TCs showed a high potential to proliferate when SEB was added (66.94 ± 2.6%). However, addition of LPS did not change the capability of TCs to proliferate (70.07 ± 1.8%).

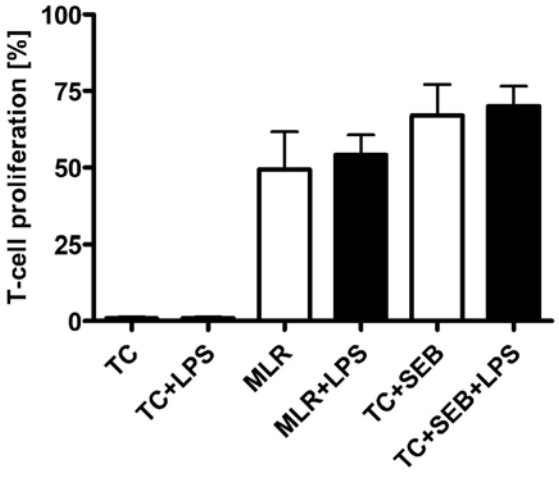

Figure 19: Alloreaction of peripheral myeloid dendritic cells and T_H0. Human mDCs were co-cultured in a MLR together with naïve TCs for 5 days in the absence (white bars) or presence (black bars) of LPS. Proliferation was assessed by CFSE staining. Bar graphs summarize data from at least 14 experiments. Bars represent means + SEM. TC – T cell, LPS – Lipopolysaccharide, MLR – mixed lymphocyte reaction (mDC + TC), SEB - Staphylococcal enterotoxin B.

Activated T_H0 cells will differentiate into T_H1 or T_H2 cells depending on their cytokine environment. Therefore, the observed effective stimulation of naïve TCs by human mDCs was further characterized by analyzing the produced cytokine environment of the resulting effector TCs. In the allogenic MLR high levels of IFN-γ (26.88 ± 4.4%) could be detected. However, the secretion of T_H2 cell producing IL-4 (3.71 ± 0.6%) was lower (Figure 20B) indicating that IFN-γ drives T_H1 cell production while IL-4 inhibits T_H1 cell production. Conversely, IL-4 drives T_H2 cell production and IFN-γ inhibits T_H2 cells. TCs in the presence with SEB alone secrete less IL-4 (1.1 ± 0.2%) and IFN-γ (12.9 ± 2.9%) (Figure 20A).

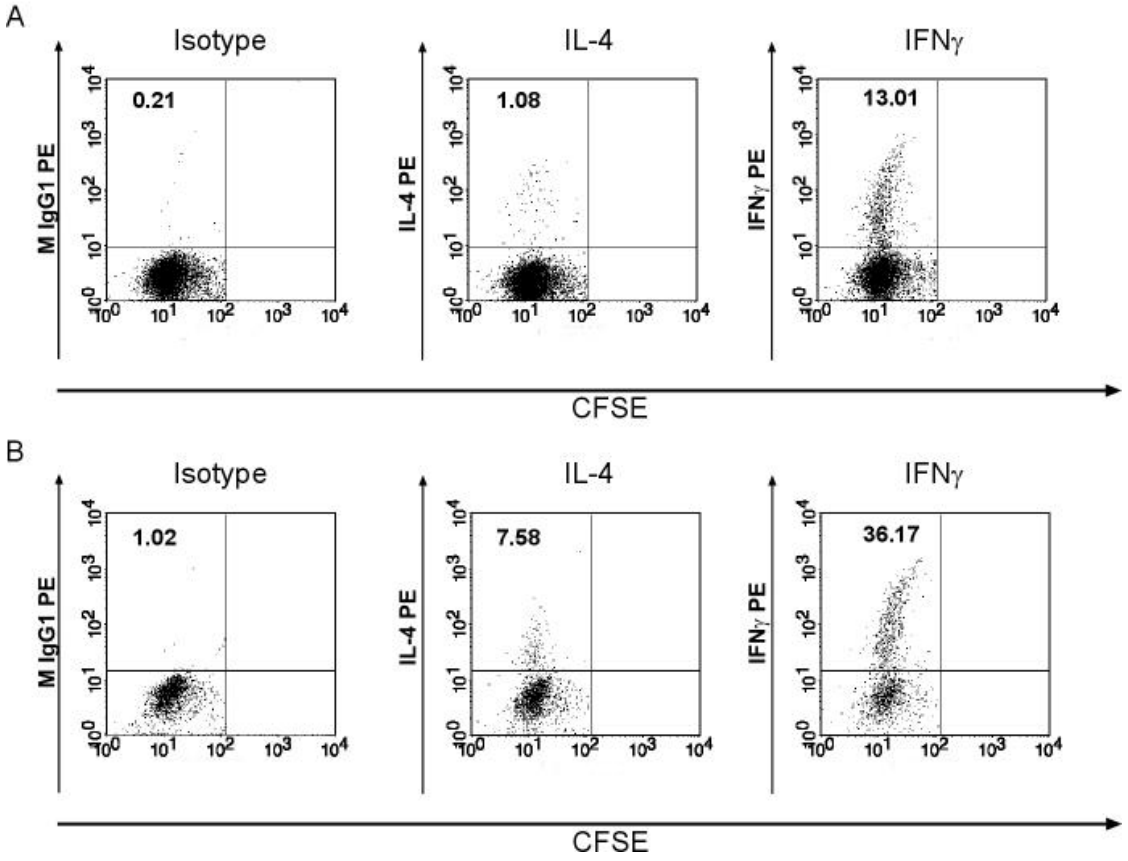

Figure 20: Mixed lymphocyte reaction of myeloid dendritic cells and naïve T cells leads to a proliferation and differentiation into T_H1 and T_H2 cells. Dot plots show representative intracellular staining for IL-4 and IFN-γ of gated proliferating TCs. Quadrant thresholds were placed determined according to isotype controls. (A) Naïve TCs cultured in the presence of SEB served as controls. (B) MLR of TCs and mDCs.

Taken together, it could be shown that human mDC effectively stimulate naïve TC in an allogenic MLR and that the proliferated cells may differentiate into effector TC.

4.2.4 Increased LPS uptake by myeloid dendritic cells in IBD patients

Since the previous experiments revealed an activated phenotype and an inflammatory response of mDCs from IBD patients to LPS could be shown, the next step was to investigate the uptake of LPS by mDCs as this could contribute to their aberrant response. In a time trail an increased LPS uptake was noticed beginning 5 hrs after incubation with LPS-Alexa 488 (Figure 21).

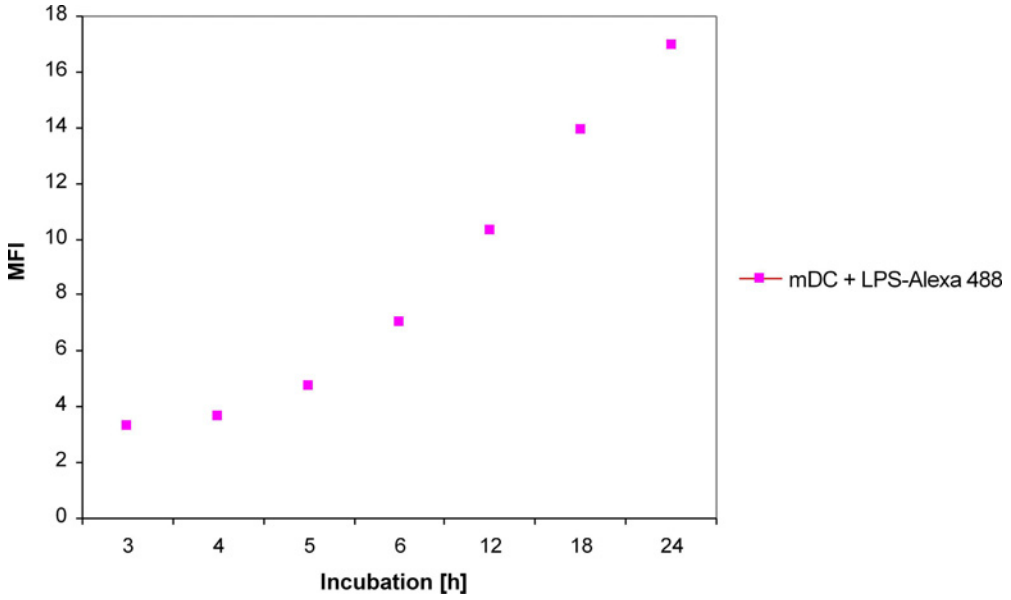

Figure 21: Kinetics of LPS uptake by myeloid dendritic cells. Human mDCs from healthy controls were incubated for the indicated time points in the presence of LPS-Alexa 488. Dots show values of mean fluorescence intensity (MFI) of healthy mDCs in the presence of LPS which were adjusted by the auto-fluorescence of mDCs alone.

Due to these results, five time points (3, 6, 12, 18 and 24 hrs) were chosen to further investigate the level of LPS uptake by mDCs. In another experiment it could be shown that this assay detects intracellular LPS only. Therefore, extracellular fluorescence was quenched with trypan blue before FACS analysis. No differences in the uptake of LPS could be noticed between quenched and not-quenched approaches. During all time points the level of assimilated LPS was similar (Figure 22).

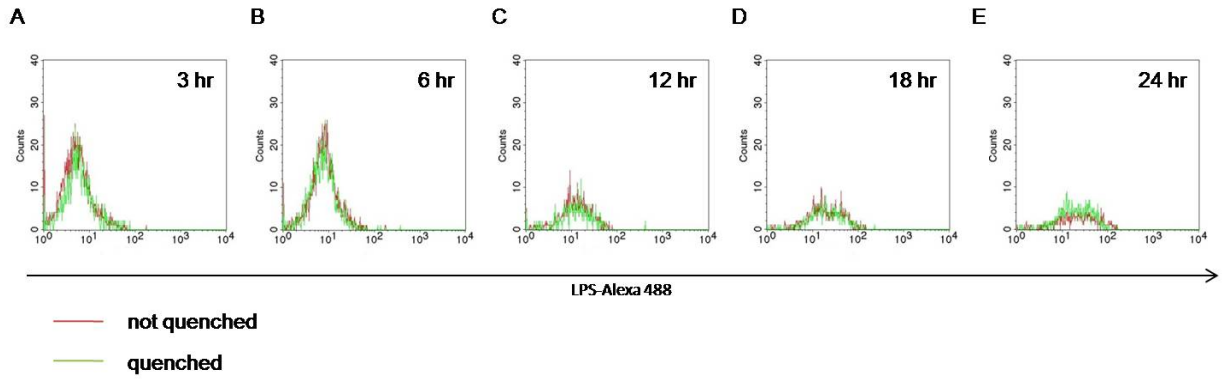

Figure 22: Comparison between quenched and unquenched approaches. LPS uptake was measured via FACS. Human mDCs were incubated for indicated time points with culture medium supplemented with Alexa Fluor 488 conjugated LPS. Afterwards, approaches were either quenched with a 0.4% trypan blue solution or directly washed without quenching the fluorescence signal. No differences in the uptake of LPS were observed between quenched and unquenched samples.

Since no differences could be observed between quenched and unquenched samples the experiment was perpetuated with healthy controls as well as IBD patients without quenching. mDCs from UC patients in RM showed an increased LPS uptake compared with healthy controls starting at approximately 18 hrs of culture. This effect continues until a culturing time of 24 hrs (Figure 23). Interestingly, mDCs from flaring UC patients showed a significant difference in the increased LPS uptake when compared to controls which is apparent already at 3 hrs and continues until 24 hrs. The same trend could be noticed in patients suffering from CD. Overall, mDCs from CD patients took up slightly more LPS than their counterparts in UC. It could be observed that mDCs from CD patients in RM showed a similar uptake of LPS compared to healthy controls over the entire 24 hrs culture period. In mDCs from flaring CD patients the increased LPS uptake compared with healthy controls was apparent already at 3 hrs. The observed effect became statistical significant after 24 hrs.

Taken together, the data correlates with the distinct response of mDCs from IBD patients to LPS and it could be shown that patients that reside in an active stage of IBD take up more LPS than healthy persons.

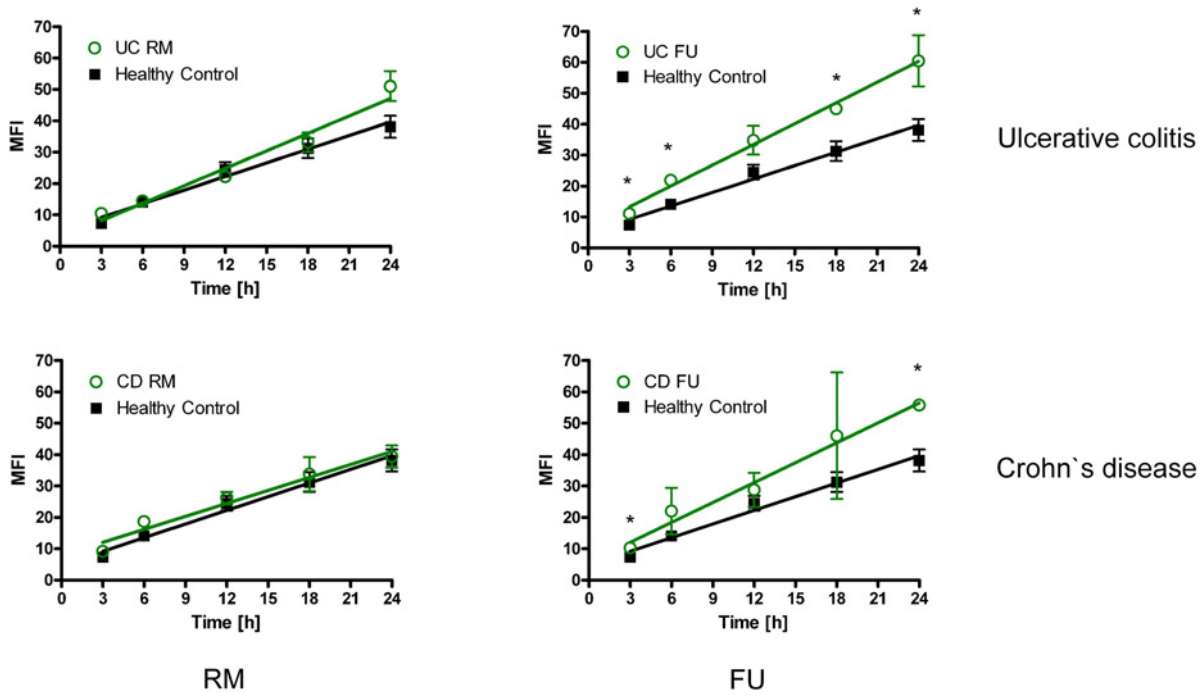

Figure 23: Increased LPS uptake by myeloid dendritic cells in active IBD. mDCs from patients with active IBD take up more Alexa 488 labeled LPS than healthy controls. Mean fluorescence intensity (MFI) was assessed over 24 hrs by FACS. Six UC and CD patients as well as six healthy controls were studied and data from five time points were analyzed. At linear regression analysis of analyzed time points all curves show an excellent quality of fit ($r^2 > 0.95$). Asteriks denote statistical significance $p < 0.05$ = *.

4.2.5 Increased TLR2 and TLR4 expression by myeloid dendritic cells from IBD patients

Innate immune responses to LPS are mediated mainly by TLR4 [156]. However, since even highly purified LPS may be contaminated with endotoxin the expression of TLR2 was also addressed in this project [157]. Both, engagement of TLR4 and TLR2, can mediate the production of inflammatory cytokines [3;158]. Thus, the expression profiles of the two receptors by mDCs were analyzed using real-time PCR on mRNA level (Figure 24). Relative levels of TLR expression were normalized by defining a value of 1 as the expression level of healthy controls. Observed relative expression levels above a value of 1.5 were assumed to show a real change in the expression level.

Most interesting were the results by freshly isolated mDCs. Here, the expression level of both receptors was higher for all IBD patients which were investigated. However, only the expression of TLR2 showed a significant increase in patients suffering from UC. Here,

patients in RM (2.1 ± 0.27) and during FU (1.9 ± 0.07) showed a significant increased relative expression of TLR2 compared to controls (Figure 24A left panel). No differences were detected between healthy controls and CD patients. On the other hand, the relative expression level of TLR4 was significantly increased in both internal diseases during an acute FU. Here, UC patients (2.4 ± 0.03) as well as patients suffering from CD (1.9 ± 0.07) showed higher expression levels of TLR4 compared with both patients in remission as well as healthy controls (Figure 24A right panel). Patients during RM state of disease did not show changes in their relative TLR4 expression level.

Furthermore, the expression of TLR2 and 4 was analyzed after culturing for 3 hrs in the absence (Figure 24B) or presence of LPS (Figure 24C) as a stimulus for mDCs. The relative expression level of TLR2 did not show any significant differences for culturing in the absence or presence of LPS as it was always lower for all patients during all stages of the disease compared to healthy controls. However, when culturing mDCs without LPS only flaring patients suffering from UC (0.14 ± 0.08) showed a significant drop in their relative expression level of TLR2. In the presence of LPS only CD patients showed a significant decrease in their TLR2 expression level whereas patients during an acute FU (0.21 ± 0.03) express less TLR2 compared to patients in RM (0.35 ± 0.06).

TLR4 expression in the absence of LPS did not show any significant changes (Figure 24B right panel). UC patients showed a similar expression pattern as healthy controls. A comparison between these patients in RM (0.82 ± 0.15) and during FU (0.97 ± 0.05) showed that they express nearly the same amount of TLR4. Interestingly, in patients suffering from CD an increase in the relative expression level (2.16 ± 0.54 for RM, 1.48 ± 0.18 for FU) could be observed in comparison to controls. However, this effect was not significant. The addition of LPS showed that only UC patients during FU (1.8 ± 0.04) and CD patients in a RM state of disease (1.5 ± 0.12) express significantly more TLR4 as their counterparts showed a similar level than healthy controls.

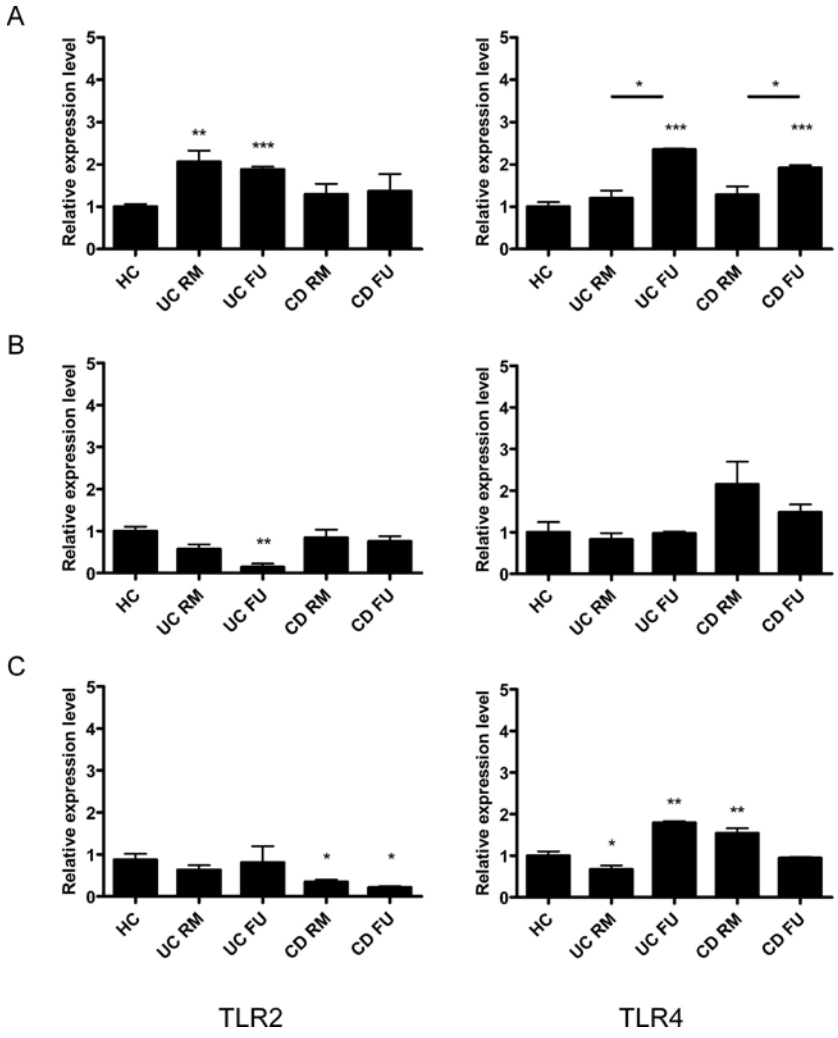

Figure 24: TLR2 and TLR4 expression by myeloid dendritic cells. (A) Freshly isolated, (B) cultured without any stimuli and (C) LPS stimulated human mDCs from healthy controls, patients with UC and CD were analyzed for the mRNA expression levels of TLR2 and TLR4 by using real-time RT-PCR. Bar graphs summarize data from at least 5 patients or controls in each category. Controls (HC) were normalized to a relative expression level of 1. HC - Healthy Control, RM – Remission, FU - Flare-up, UC - Ulcerative Colitis, CD - Crohn's Disease. Asterisks denote statistical significance $p < 0.05$= *, $p < 0.01$= **, $p < 0.001$ = ***.

4.3 Effects of the probiotic yeast *Saccharomyces boulardii* on human myeloid dendritic cells

Since the treatment with probiotics seems to be effective in patients with IBD the aim of this part of thesis was the investigation of the anti-inflammatory potential of the probiotic yeast *Sb* in humans.

4.3.1 *Saccharomyces boulardii* culture supernatant decreases the number of CD40, CD80 and CCR7 positive myeloid dendritic cells after incubation with LPS in healthy controls

Based on the previous observation showing that more mDCs from healthy controls express CD40 and CD80 when cultured in the presence of LPS, the following experiments addressed the question whether the probiotic yeast *Sb* has an effect on human mDCs and their control of TC activation. It has been demonstrated in controlled clinical trials by several groups that *Sb* is effective in inflammatory and infectious disorders of the gastrointestinal tract [119-124]. However, little is known of how this yeast unfolds its anti-inflammatory properties in human.

After stimulation with LPS and incubation for 21 hrs in the presence or absence of *Sb*S in different dilutions (1:2, 1:8, 1:32) mDCs were stained for the two co-stimulatory molecules CD40 and CD80 as well as for the maturation markers CD83, CD83 and CD197 (CCR7).

The addition of *Sb*S to mDCs in culture experiments significantly decreased the number of CD40 and CD80 expressing mDCs (Figure 25A, B). Comparison between CD40 expressing cells in the presence or absence of *Sb*S showed significant differences in the percentage of CD40 positive mDCs for *Sb*S dilutions of 1:2 and 1:8. However, a dilution of 1:32 did not show any significant differences. The same trend could be observed for the co-stimulatory molecule CD80. Here, the number of positive mDCs was significantly lower compared to culture approaches with LPS alone when *Sb*S was added to dilutions of 1:2 and 1:8 but not 1:32.

While freshly isolated mDCs express virtually no CCR7, stimulation with LPS induced an expression of CCR7 by virtually all mDC positive cells (Figure 25C). Furthermore, addition of *Sb*S to these approaches significantly decreased the number of CCR7 expressing mDCs for *Sb*S dilutions of 1:8 and 1:32.

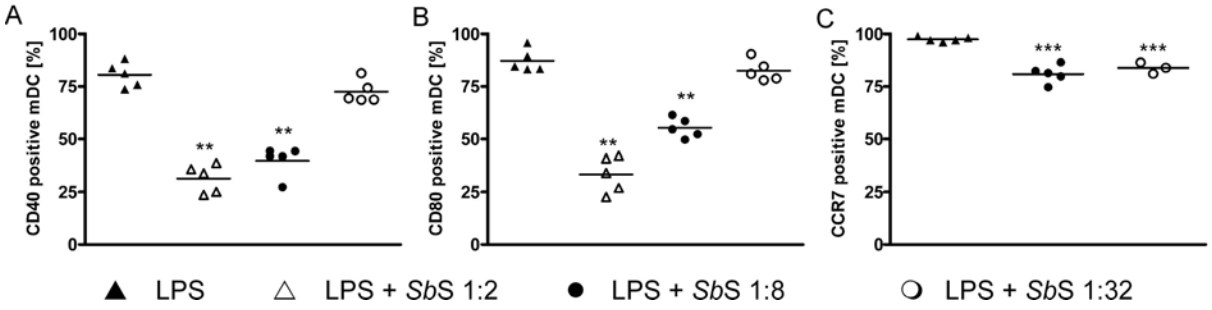

Figure 25: *Sb*S reduces the number of activated myeloid dendritic cells. *Sb*S decreases the number of (A) CD40, (B) CD80, and (C) CCR7 positive when added to cultured mDCs together with LPS compared with LPS alone. This effect is dose-dependent. The results represent data from five independent experiments with healthy controls. Asteriks denote statistical significance $p < 0.01$ = **, $p < 0.001$ = ***.

In addition to the previous findings two other important DC maturation markers were studied. The glycoprotein CD83 is one of the best-known maturation markers for human DCs. It is known that CD83 is strongly upregulated during DC maturation along with other co-stimulatory molecules such as CD80 and CD86. This suggests an important role of CD83 in the induction of immune responses. It could be confirmed that stimulation of mDCs with LPS induced an expression of CD83 as well as CD86 by virtually all mDC positive cells (Figure 26). However, the addition of *Sb*S dilutions 1:8 and 1:32 did not show any differences in the number of CD83 and CD86 positive mDCs.

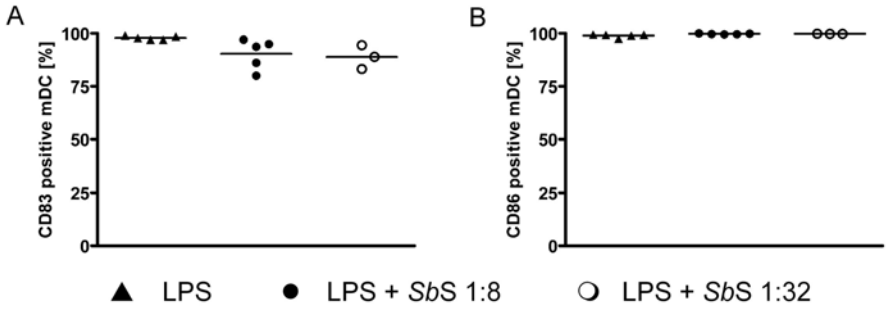

Figure 26: *Sb*S has no effects on the number of CD83 and CD86 positive myeloid dendritic cells. No changes could be observed in the number of (A) CD83 and (B) CD86 positive mDCs when *Sb*S was added in different dilutions to cultured mDCs together with LPS compared with LPS alone. The results represent data from five or three independent experiments with healthy controls.

4.3.2 *Saccharomyces boulardii* culture supernatant reduces secretion of TNF-α and IL-6 and increases secretion of IL-10 by myeloid dendritic cells

To further investigate the cytokine secretion by LPS stimulated mDCs in the presence or absence of *Sb*S the cell culture supernatants from the previous experiments were analyzed. Therefore pro-inflammatory cytokines such as TNF-α and IL-6 as well as the anti-inflammatory cytokine IL-10 were measured by CBA (Figure 27). Since the cytokine secretion of mDCs after stimulation with LPS showed a wide variation in the pg/ml range (228.2 pg/ml to 933.4 pg/ml for TFN-α, 661.6 pg/ml to 5365.0 pg/ml for IL-6 and 150.8 pg/ml to 2030.0 pg/ml for IL-10) the controls were normalized to 100% and the increase or decrease of cytokine secretion depending on *Sb*S was further investigated. Furthermore, a substantial cell death was observed when adding 1:2 diluted *Sb*S to LPS stimulated mDCs. Therefore, only *Sb*S dilutions of 1:8 and 1:32 were considered for analysing this set of experiments.

First, the production of IL-6 was studied. It could be observed that LPS stimulated mDCs secreted significantly more IL-6 than samples with supplemental *Sb*S. This effect was noticed for both dilutions of the yeast supernatant which were used in this experiment (56.01 ± 11.32% for *Sb*S 1:8 and 57.54 ± 6.95% for *Sb*S 1:32) (Figure 27A). Since DCs are known to have a dual role in the process of inflammation and they may also help to control inflammation independent of regulatory TCs, the next step was to look at the secretion of IL-10 by mDCs in the presence of *Sb*S [23;155]. LPS stimulated mDCs showed to secrete significantly more IL-10 when *Sb*S was added in a dilution of 1:8 (161.90 ± 16.96%). This effect was dose dependent as a further significant increase in the production of IL-10 could be observed when *Sb*S was used in a higher dilution of 1:32 (204.40 ± 10.98%) (Figure 27B). Further, the secretion of TNF-α by LPS stimulated mDCs decreased significantly when *Sb*S was added to the culture at dilutions of 1:8 to 48.42 ± 10.43% and 1:32 to 38.91 ± 7.73% which echoed the results seen in the secretion of IL-6 (Figure 27C).

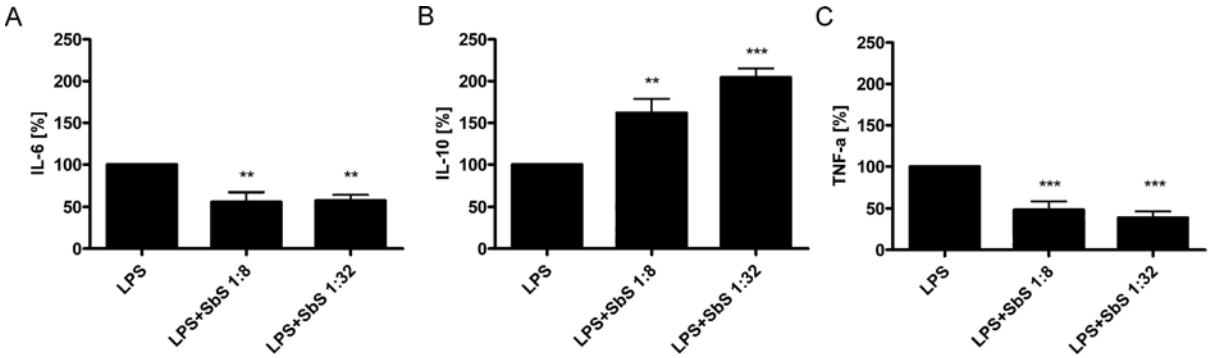

Figure 27: Cytokine secretion of human myeloid dendritic cells after stimulation with LPS and *Sb*S. *Sb*S reduces the secretion of (A) IL-6 and (C) TNF-α and shows an increase in the secretion of anti-inflammatory (B) IL-10. Bar graphs express means + SEM from five independent experiments with healthy controls. mDC cultured with LPS alone were normalized to 100%. Asteriks denote statistical significance $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

4.3.3 *Saccharomyces boulardii* culture supernatant inhibits T cell proliferation in an allogenic mixed lymphocyte reaction

Since the first set of experiments suggested an effect of *Sb*S on co-stimulatory molecules and DC maturation markers which are highly relevant for TC activation, its direct effect on TC proliferation was investigated. Therefore, the effect in an allogenic MLR of naïve TCs and mDCs was studied in this thesis (Figure 28). Naïve TCs showed less auto-proliferation after stimulation with LPS alone (2.47 ± 1.21%). However, in an allogenic MLR in which mDCs were stimulated with LPS a robust proliferation of 36.11 ± 2.46% could be observed. The addition of *Sb*S at a dilution of 1:32 revealed no significant effect (35.01 ± 2.28%) whereas a dilution of 1:8 significantly reduced proliferation to 16.40 ± 2.75% (Figure 28C). Additional dilution of *Sb*S to 1:2 induced an increased cell death of mDCs resulting in unacceptable variances of the TC/DC ratio and was therefore omitted from the analysis.

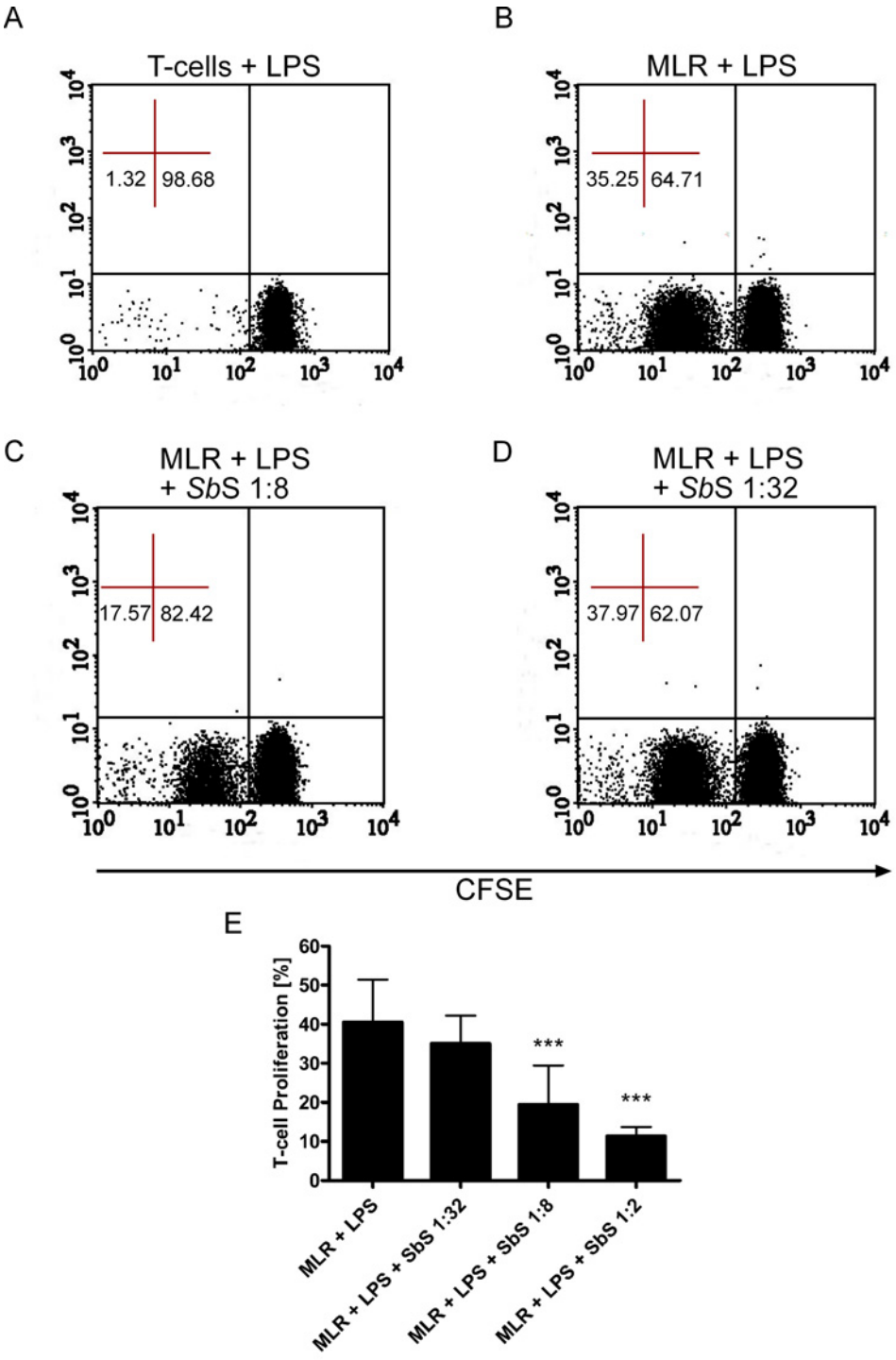

Figure 28: *Sb*S inhibits T cell activation and proliferation in an allogenic mixed lymphocyte reaction with myeloid dendritic cells. FACS plots from representative proliferation experiments assessed by CFSE staining. (A) Naïve TCs cultured without mDCs in the presence of LPS alone served as controls. (B) MLR of TCs and mDCs preincubated with LPS show a robust proliferation in the absence of *Sb*S. (C) Adding *Sb*S to the MLR results in a significant decline of TC proliferation for a *Sb*S dilution of 1:8. (D) Further dilution of *Sb*S to 1:32 could not maintain a significant inhibitory effect on TC proliferation. (E) Data from these experiments is summarized in bar graphs (n=10). Bars represent means + SEM. Asterisks denote statistical significance $p < 0.001$ = ***.

4.3.4 *Saccharomyces boulardii* culture supernatant diluted 1:2 induced substantial cell death

Since the MLR experiments as well as the measurement of important activation marker showed a massive cell death when *Sb*S at a dilution of 1:2 (49.48 ± 5.91%) was used, further experiments were carried out without this set of dilution. Dilutions of 1:8 (19 ± 1.5%) and 1:32 (20.67 ± 1.0%) showed a normal cell death ratio which was also observed with approaches just containing LPS (20.94 ± 1.5%) as a stimulus (Figure 29).

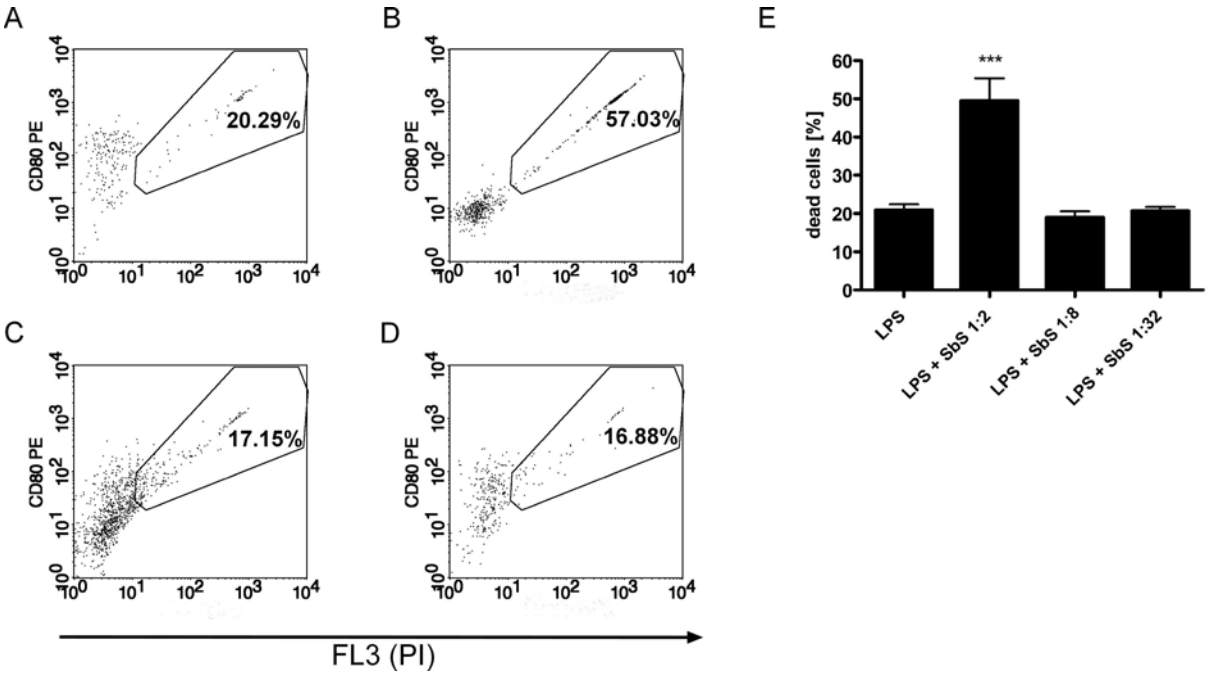

Figure 29: Number of dead cells in experiments with *Sb*S. FACS plots from representative CD80 stainings. (A) mDCs in the addition of LPS. (B) The addition of *Sb*S in a 1:2 dilution increases the number of dead viable cells. (C) Further dilution of *Sb*S to 1:8 and (D) 1:32 show a similar pattern of dead cells as the control approach containing mDCs and LPS. Dead cells were counted by addition of propidium iodide. The bar graphs (E) summarize the results. Bars represent means + SEM. PI – Propidium Iodide. Asterisks denote statistical significance $p < 0.001$ = ***.

4.3.5 The active component in *Saccharomyces boulardii* culture supernatant has a molecular weight of <3 kDa

The production of *Sb*S was done without the use of a 10 kDa cutoff filter as it was described in the literature, to avoid exclusion of potentially biologically active larger molecules [138]. Therefore, the next aim was to determine the approximate molecular weight of the active

component of *Sb*S by repeating the phenotypic and cytokine secretion studies with *Sb*S permeates (diffusates) ranging from <100 kDa down to <3 kDa.

4.3.5.1 *Saccharomyces boulardii* culture supernatant permeates reduce the number of CD40, CD80 and CCR7 positive myeloid dendritic cells

Compared with LPS stimulated mDCs alone, the presence of 1:8 diluted *Sb*S and all tested permeates in these cultures significantly decreased the number of CD40, CD80 and CD197 (CCR7) expressing mDCs (Figure 30).

First of all, the two maturation markers CD83 and CD86 were studied. Here, no differences in the number of CD83 and CD86 positive mDCs could be observed when the different permeates of *Sb*S were added. All approaches showed nearly the same high amount of CD83 and CD86 positive mDCs. This finding is line with previous observations that the addition of *Sb*S has no effect on the number of positive mDCs.

Further on, all *Sb*S permeates significantly reduced the percentage of CD40 expressing healthy mDCs in comparison to LPS only stimulated cells (Figure 30C). The same trend was observed for CD80. Here, permeates significantly reduced the percentage of expressing mDCs at all stages of fractionation (Figure 30D). Finally, it could be also shown that all yeast permeates significantly reduced the percentage of CD197 expressing mDCs as well (Figure 30E).

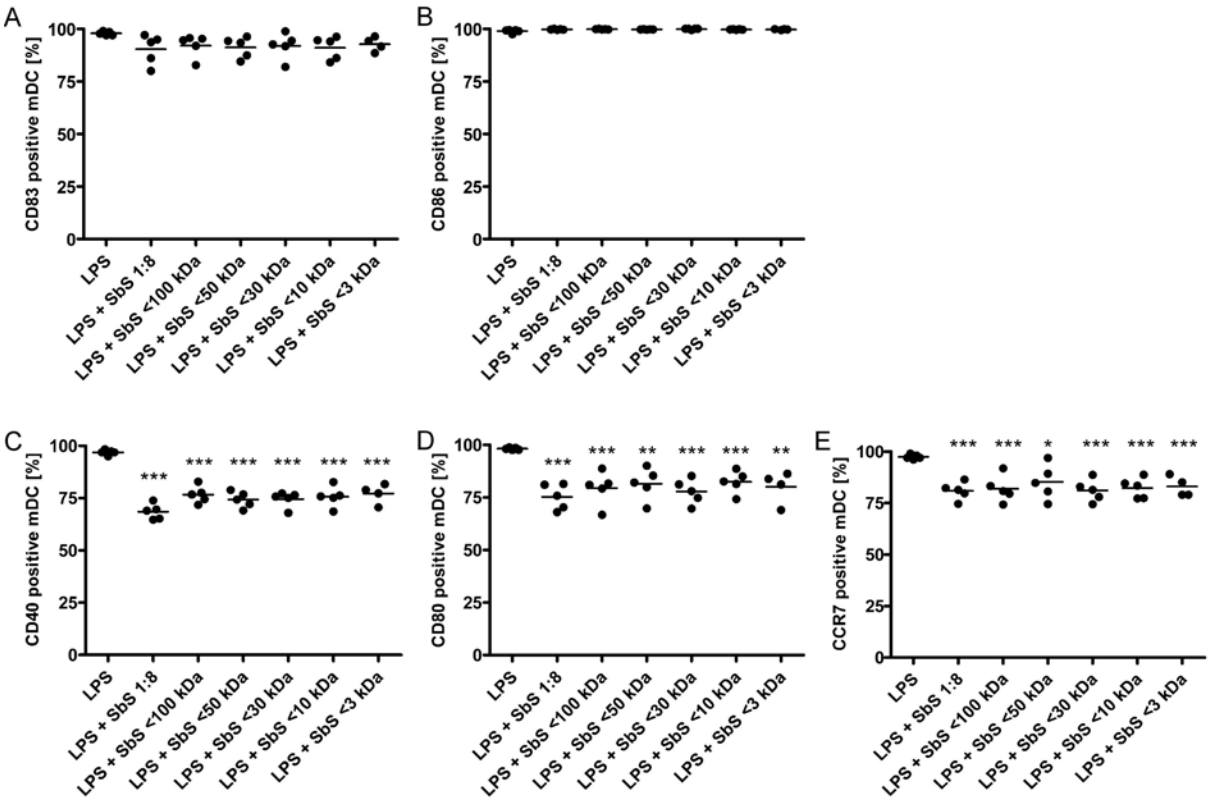

Figure 30: Expression of co-stimulatory molecules and maturation marker in the presence of *Sb*S permeates. Compared with LPS stimulated mDCs alone, the presence of 1:8 diluted *Sb*S and *Sb*S permeates ranging from <100 kDa down to <3 kDa in these cultures significantly decreased the number of (C) CD40, (D) CD80 and (D) CD197 (CCR7) expressing mDCs. The two maturation markers (A) CD83 and (B) CD86 do not show any changes in the number of positive mDCs. The results represent data from five independent experiments. sp < 0.05 = *, p < 0.01 = **, p < 0.001 = ***.

Taken together, the fractionation strategie of the supernatant of *Sb* and further investigation in the expression of important co-stimulatory molecules and maturation marker showed no differences in using a 1:8 dilution of the supernatant or in using any of the permeates indicating that the active component in *Sb*S must have a molecular weight smaller than 3 kDa.

To further corroborate this data, two retentates of the ultrafiltration process (Ret 100 kDa, Ret 3 kDa) were used and identical experiments were performed. Interestingly, the opposite results could be assessed (Figure 31). The retentates did not induce the phenotypic changes and cytokine secretion pattern which could observed previously with the according *Sb*S permeates. These results suggests, that the active component of the yeast is included in the permeates.

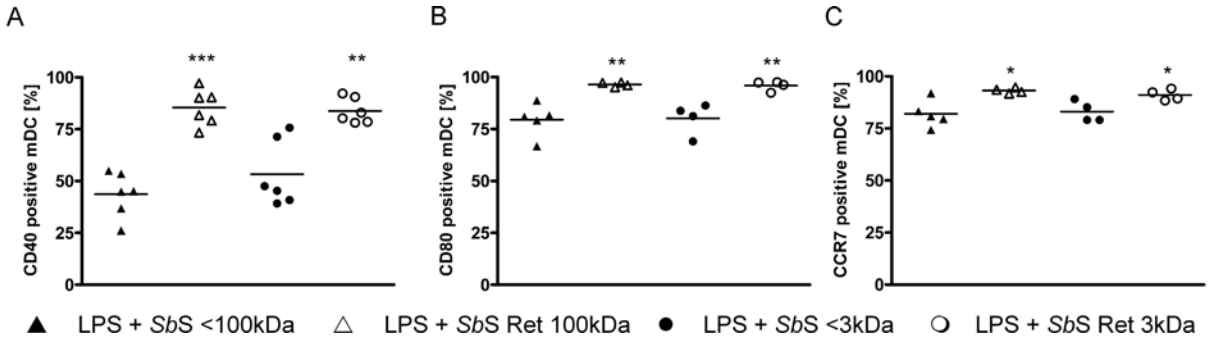

Figure 31: Expression of co-stimulatory molecules and maturation marker in the presence of *Sb*S retentates. The retentates from the ultrafiltration process show an increase in the number of (A) CD40, (B) CD80 and (C) CCR7 expressing mDCs. The results represent data from 4-5 independent experiments. Ret – Retentate. $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

In summary, all yeast permeates ranging from <100 kDa down to <3 kDa resulted in a similar phenotype and cytokine secretion compared with non-ultrafiltrated *Sb*. The results were further supported by the additional results with the yeast retentates. Taken together, the active component in *Sb*S appears to have a molecular weight smaller than <3 kDa.

4.3.5.2 *Saccharomyces boulardii* culture supernatant permeates have an effect on cytokine secretion

Compared with LPS stimulated mDCs alone, the presence of 1:8 diluted *Sb*S and all tested yeast permeates in these cell cultures significantly decreased the secretion of TNF-α and IL-6 and increased the secretion of IL-10 (Figure 32). Due to a wide variation within the measurement of cytokine levels (pg/ml), which were mentioned above, the controls were normalized to 100% and the increase or decrease of cytokine secretion depending on *Sb*S permeates was investigated.

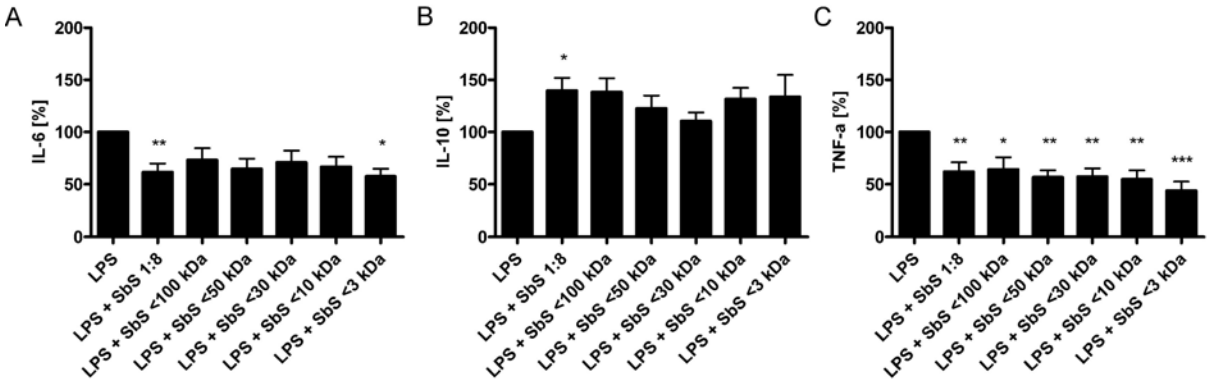

Figure 32: *Sb*S permeates reduce secretion of IL-6 and TNF-α and show an increase in the secretion of IL-10. Compared with LPS stimulated mDCs alone, the presence of 1:8 diluted *Sb*S and *Sb*S permeates ranging from <100 kDa down to <3 kDa in these cultures significantly decreased the secretion of (A) IL-6 and (C) TNF-α and shows an increase in the secretion of anti-inflammatory (B) IL-10. Bar graphs show means + SEM from five independent experiments with healthy controls. mDC cultured with LPS alone were normalized to 100%. Asteriks denote statistical significance $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

All *Sb*S permeates significantly reduced the secretion of pro-inflammatory TNF-α by mDCs compared to LPS stimulated only mDCs (Figure 32C). The same trend could be observed for IL-6. Here, all approaches resulted in a lower level of secreted IL-6. However, only the permeate smaller than 3 kDa showed a significant reduction of IL-6 (Figure 32A). In contrast to the observed decrease in the secretion of the two key pro-inflammatory cytokines TNF-α and IL-6, the addition of *Sb*S permeates led to an increase in the production of IL-10 by mDCs (Figure 32B).

4.4 Effects of *Saccharomyces boulardii* on human myeloid dendritic cells from IBD patients

Having demonstrated that *Sb* inhibits LPS-induced activation of human mDCs in healthy volunteers the next step was to examine the effects of this yeast preparation on mDCs which were isolated from patients. Therefore, mDCs from patients were phenotyped by their expression of typical activation markers and co-stimulatory molecules. Furthermore, the production of key cytokines was evaluated in the presence of *Sb*S after stimulating the cells with LPS.

To rule out a specific effect only on patients suffering from UC and CD an additional control group was added in this study. Patients with an infectious colitis (IC) were also assessed in this study.

4.4.1 *Saccharomyces boulardii* culture supernatant reduces the number of CD40, CD80 and CCR7 positive myeloid dendritic cells in patients with Ulcerative Colitis and Crohn's Disease following LPS stimulation

To examine whether the yeast has also an effect on DCs from IBD patients the phenotype of these cells was studied in the presence or absence of *Sb*S following stimulation with LPS.

The addition of *Sb*S to LPS stimulated mDCs from patients in culture experiments significantly decreased the number of CD40, CD80 and CCR7 expressing mDCs (Figure 33 A-C). A comparison between CD40 expressing cells in the presence or absence of *Sb*S showed significant differences in the percentage of CD40 positive mDCs for all patients.

The same trend could be observed for the co-stimulatory molecule CD80, where LPS also induced a substantial expression in the number of positive cells in RM and acute FU for all groups of patients, respectively. As for CD40, the addition of *Sb*S in a dilution of 1:8 to these cultures significantly decreased the fraction of CD80 expressing mDCs during all stages of the disease.

Having previously shown, that a stimulation of mDCs from healthy volunteers with LPS induces an expression of CCR7 by virtually all mDC positive cells (Figure 25C) the next step was to investigate the expression of this migration marker for IBD and IC patients, respectively. The addition of *Sb*S to these approaches significantly decreased the number of CCR7 expressing mDCs in UC and CD patients during RM. Furthermore, IC patients also showed a drop in their number of CCR7 expressing mDCs in the presence of LPS and *Sb*S. The investigation of CD83 and CD86 showed no significant differences in the number of positive mDCs. A stimulation of mDCs with LPS induced an expression of the two molecules by virtually all positive cells. However, the addition of *Sb*S did not show any differences in the number of CD83 and CD86 positive mDCs for all investigated patients.

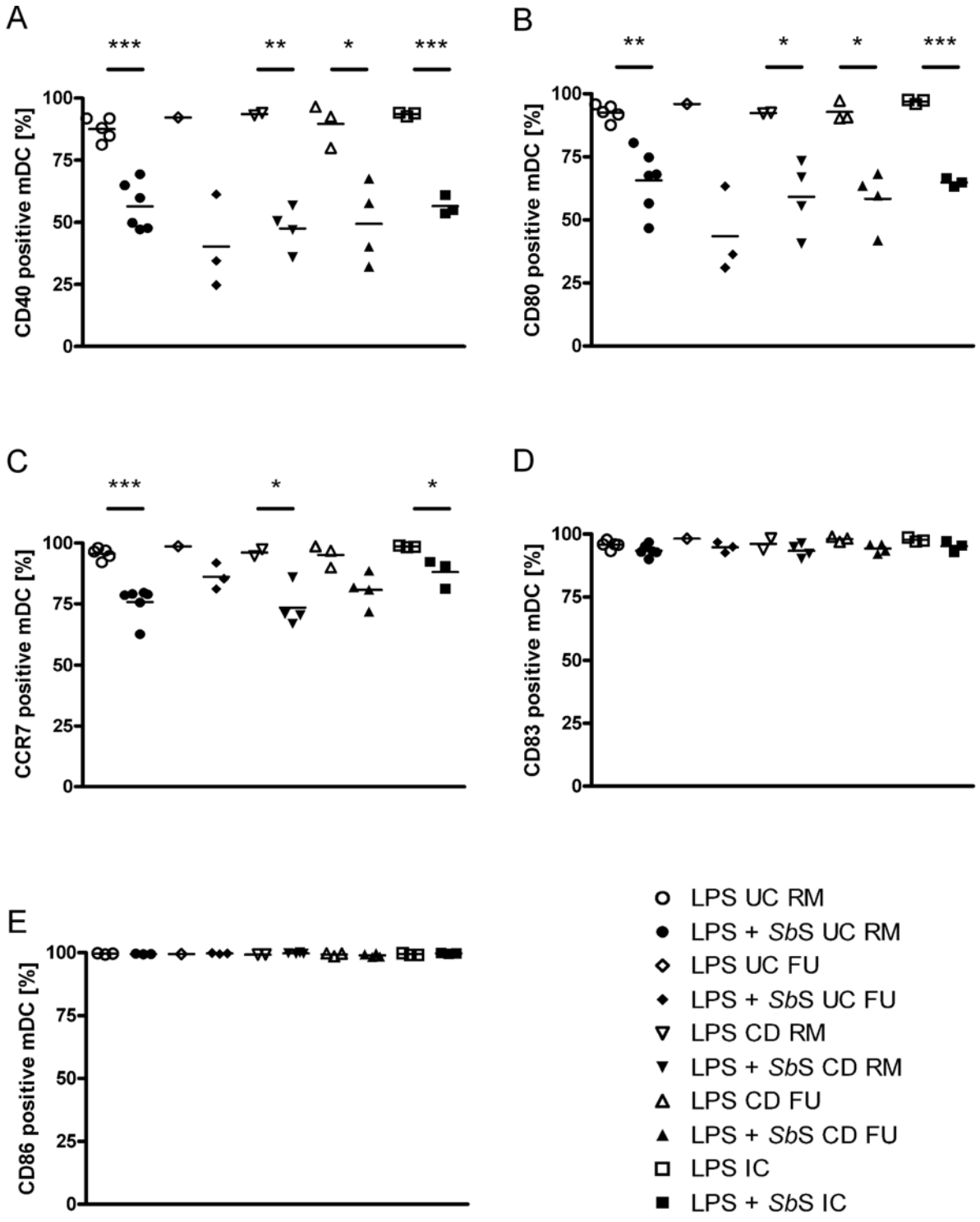

Figure 33: *Sb*S reduces the number of activated myeloid dendritic cells in patients. *Sb*S shows a decrease in the number of (A) CD40, (B) CD80, and (C) CCR7 positive cells when added to LPS stimulated mDCs in a dilution of 1:8. No changes could be observed in the number of (D) CD83 and (E) CD86 positive mDCs when *Sb*S was added to cultured mDCs together with LPS compared with LPS alone. The results represent data from at least three independent experiments for each patient category. Bars represent means ± SEM. UC – Ulcerative Colitis, CD – Crohn's Disease, IC – Infectious Colitis, RM – Remission, FU – Flare-up. Asteriks denote statistical significance $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

4.4.2 *Saccharomyces boulardii* culture supernatant reduces secretion of important pro- and anti-inflammatory cytokines by myeloid dendritic cells in IBD patients

Cell culture supernatants were also investigated for the secretion of important cytokines. Therefore, pro-inflammatory IL-6 and TNF-α as well as the anti-inflammatory IL-10 were investigated by CBA (Figure 34). As mentioned above, mDCs stimulated with LPS only were normalized to 100% to compensate for a wide variation in the pg/ml range for each measured cytokine secretion.

It could be observed that LPS stimulated mDCs secrete more IL-6 than samples with supplemental *Sb*S. This effect was noticed for IBD patients both in RM (46.61 ± 5.87% for UC, 75.94 ± 19.12% for CD) and during an acute phase of the disease (64.53 ± 11.21% for UC, 82.33 ± 12.89 for CD). However, a significant drop in the secretion of IL-6 could only be observed for UC patients during a RM stage of their disease. Patients suffering from CD showed a slightly higher secretion of IL-6 compared to UC.

Having shown that healthy mDCs secrete significantly more IL-10 when *Sb*S was added in a dilution of 1:8, the next step was to investigate the level of IL-10 secretion in IBD patients. mDCs from UC patients showed a decrease in their level of IL-10 secretion when *Sb*S was added together with LPS to culture experiments compared with LPS alone (68.65 ± 7.41% for RM, 56.72 ± 9.59% for FU). However, this effect was only significant for UC patients during an acute FU of their disease. On the other hand, patients suffering from CD showed only a slight drop during FU (80.48 ± 17.88%) whereas an increase in the secretion of IL-10 was observed during an RM state of disease (129.4 ± 32.48%).

Furthermore, the secretion of TNF-α by LPS stimulated mDCs showed also a decrease when *Sb*S was added to the culture. The two different types of IBD showed different TNF-α secretion patterns but overall a trend similar to the secretion of IL-6 was seen. Here, CD patients (98.43 ± 19.87% for RM, 60.86 ± 6.79% for FU) showed a higher secretion of TNF-α compared to UC patients (35.6 ± 7.71% for RM, 70.86 ± 13.6% for FU).

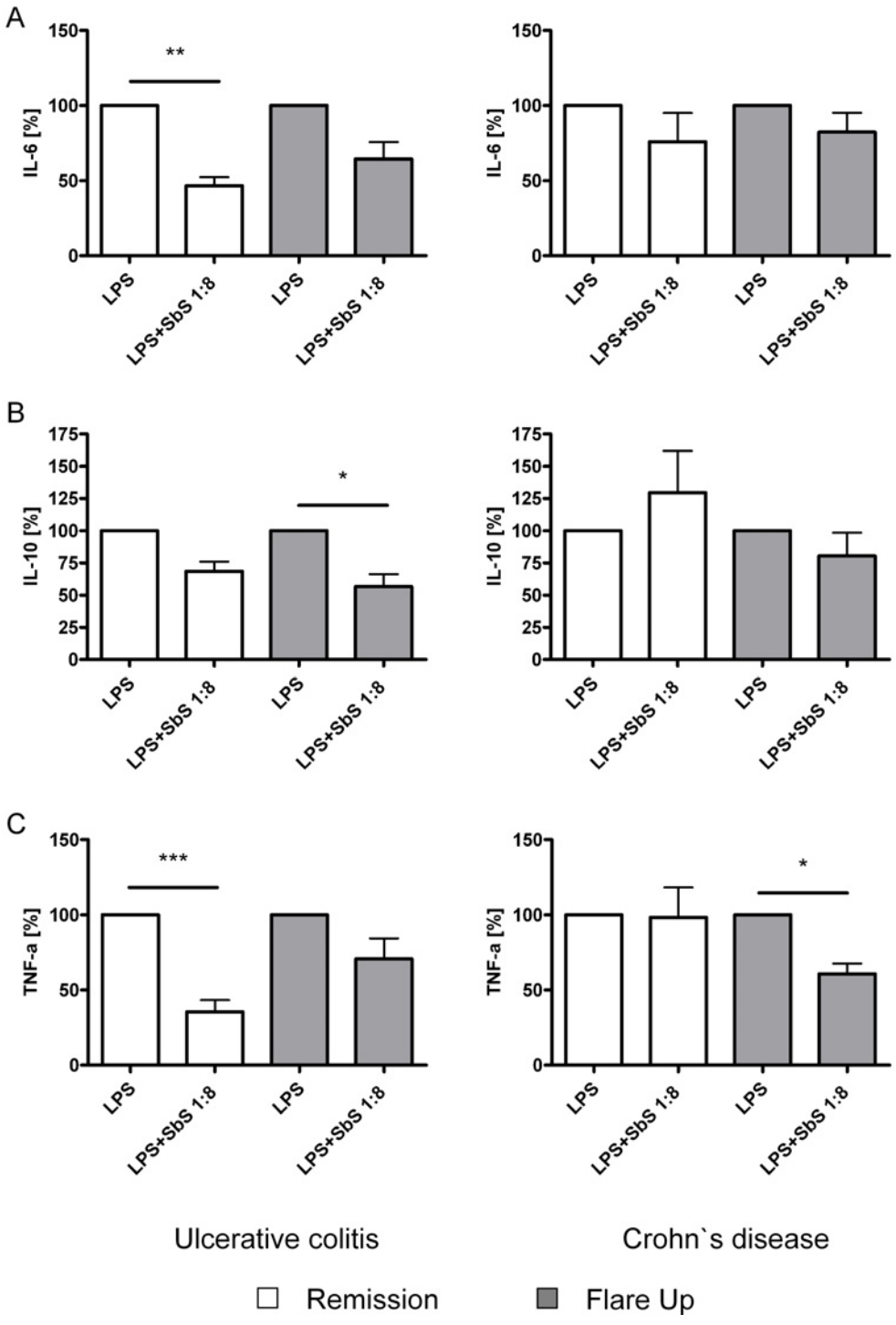

Figure 34: Cytokine secretion of myeloid dendritic cells from IBD patients after stimulation with LPS and *Sb*S. *Sb*S reduces the secretion of (A) IL-6, (B) IL-10 and (C) TNF-α in IBD patients. *Sb*S was added in a dilution of 1:8 to the cell culture. Bar graphs express means + SEM from at least three independent experiments within each group. mDC cultured alone in the presence of LPS were normalized to 100%. Asteriks denote statistical significance $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

The next step was the investigation of mDCs isolated from patients suffering from IC as another site of inflammation in the gut (Figure 35). No differences could be observed regarding the secretion of key cytokines when *Sb*S was added together with LPS (82.35 ± 14.31% for IL-6, 93.93 ± 19.15% for IL-10, 76.16 ± 16.96% for TNF-α).

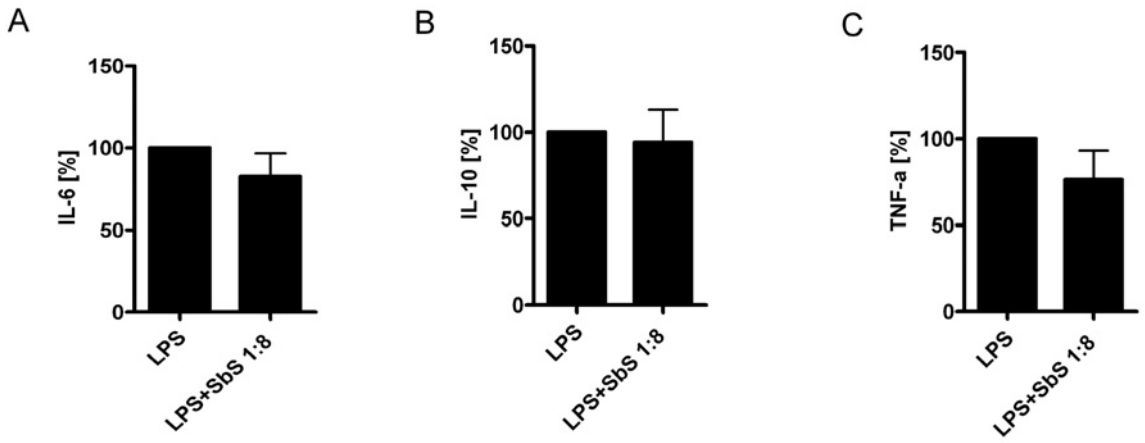

Figure 35: Cytokine secretion of myeloid dendritic cells from patients suffering from IC. *Sb*S with a dilution of 1:8 reduces the secretion of (A) IL-6, (B) IL-10 and (C) TNF-α in patients with IC. Bar graphs express means + SEM from at least three independent experiments within each group. mDCs cultured alone in the presence of LPS were normalized to 100%.

4.5 Phenotype and development of human dendritic cells in the mucosa

The previous experiments were limited on studying human blood DCs of IBD patients and healthy controls. Since our group could demonstrate that patients suffering from CD and UC experience a significant drop in their peripheral mDC populations during acute flare-ups and other groups could show similar changes in the circulating DC populations, the aim of the second part of this study was to look at DCs in the mucosa of the gut from patients with IBD and patients having other diseases and were undergoing gut surgery [144;159-161].

4.5.1 Increased frequency of activated myeloid dendritic cells among lamina propria mononuclear cells in IBD patients

It is conceivable, that circulating blood DCs in active IBD migrate to secondary lymphatic organs and other sites of inflammation. Our group has previously demonstrated that

circulation mDCs express gut homing markers on their surface. Therefore, the aim was now to evaluate DCs which were taken from colonic mucosal specimens from patients undergoing surgery for refractory IBD or from controls that underwent colonic resection for non-inflammatory conditions, e.g. diverticulosis [144]. The frequency of mDCs in the inflamed mucosa and the expression of important activation and maturation markers for mDCs were enumerated and evaluated by FACS, respectively (Figure 36).

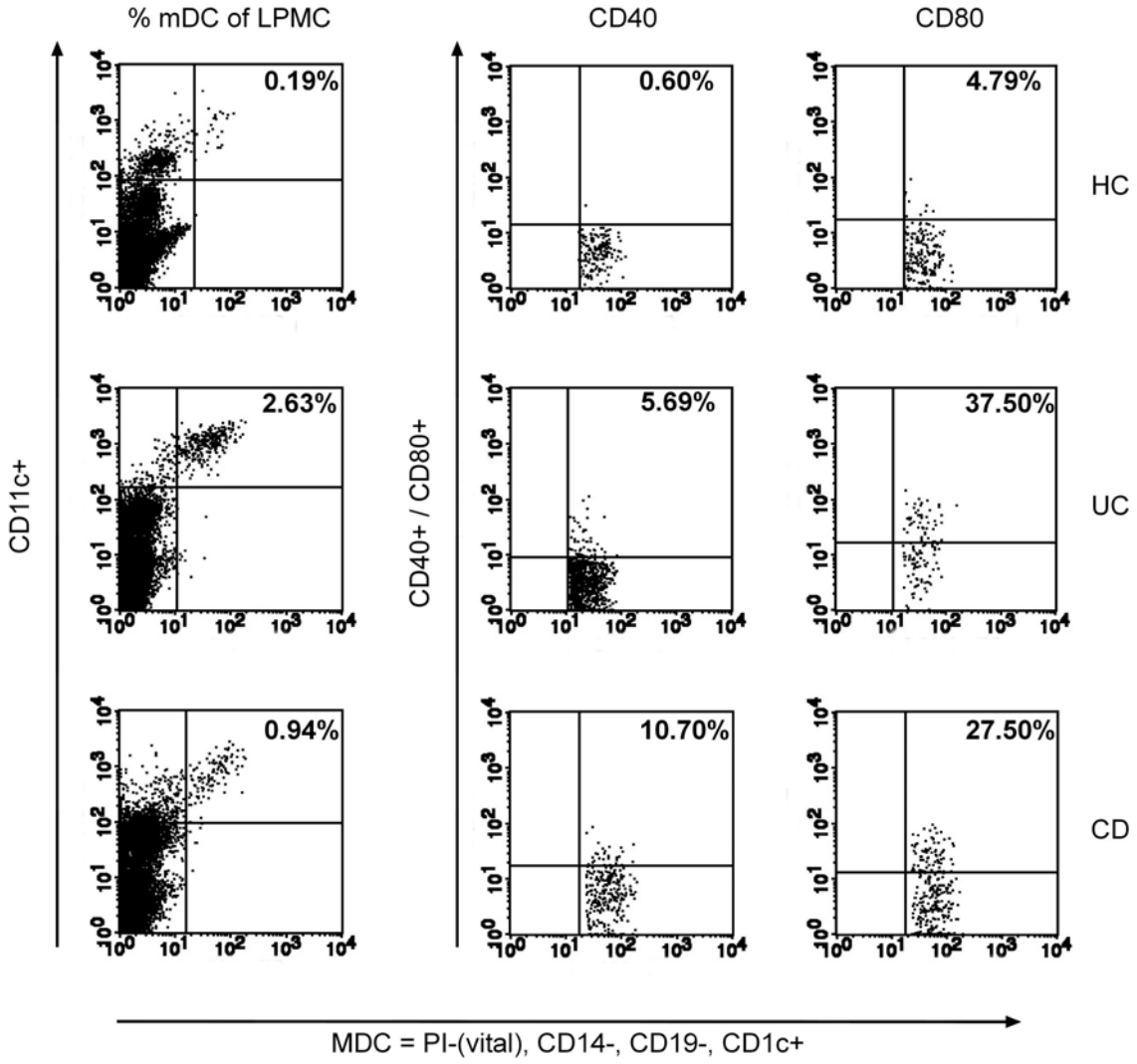

Figure 36: Increased frequency of activated myeloid dendritic cells in the mucosa. In patients with active IBD an increased frequency of mDCs is found compared to controls. More mucosal mDCs from UC and CD patients display an activated phenotype with an increased fraction of CD40 and CD80 expressing mDCs. FACS plots show representatives from 10 patients or controls within each group. Quadrant thresholds were placed according to isotype controls. HC – Healthy Control, UC – Ulcerative Colitis, CD – Crohn's Disease.

A significantly higher frequency of mDCs in the inflamed mucosa of UC patients (0.93 ± 0.57% of LPMCs) and CD patients (0.63 ± 0.18% of LPMCs) was found in comparison with tissue from non-IBD controls (0.24 ± 0.05% of LPMCs) (Figure 37A).

In a next step the expression of important DC markers was enumerated by FACS analysis. The assay showed that a significantly higher number of mucosal DCs express the two activation markers CD40 and CD80 compared with non-IBD controls. In patients suffering from UC, a higher frequency of CD40 expressing mucosal mDCs was found (7.11 ± 0.72%) when compared with non-IBD controls (0.98 ± 0.34%). The frequency of CD80 expressing mucosal mDCs in UC was 23.10 ± 7.28% vs. 6.46 ± 0.90% compared with non-IBD controls. In CD, the occurrence of CD40 (11.60 ± 0.90%) and CD80 (14.73 ± 12.77%) expressing mucosal mDCs was also higher as in non-IBD (Figure 37B, C). Furthermore, the expression of CD86 was studied. No significant differences were found when patients suffering from UC (59.67 ± 13.75%) and CD (86.15 ± 5.85%) were compared with non-IBD controls (81.64 ± 2.68%). However, there was a trend showing a lower frequency of CD86 expressing mDCs in the mucosa of UC patients.

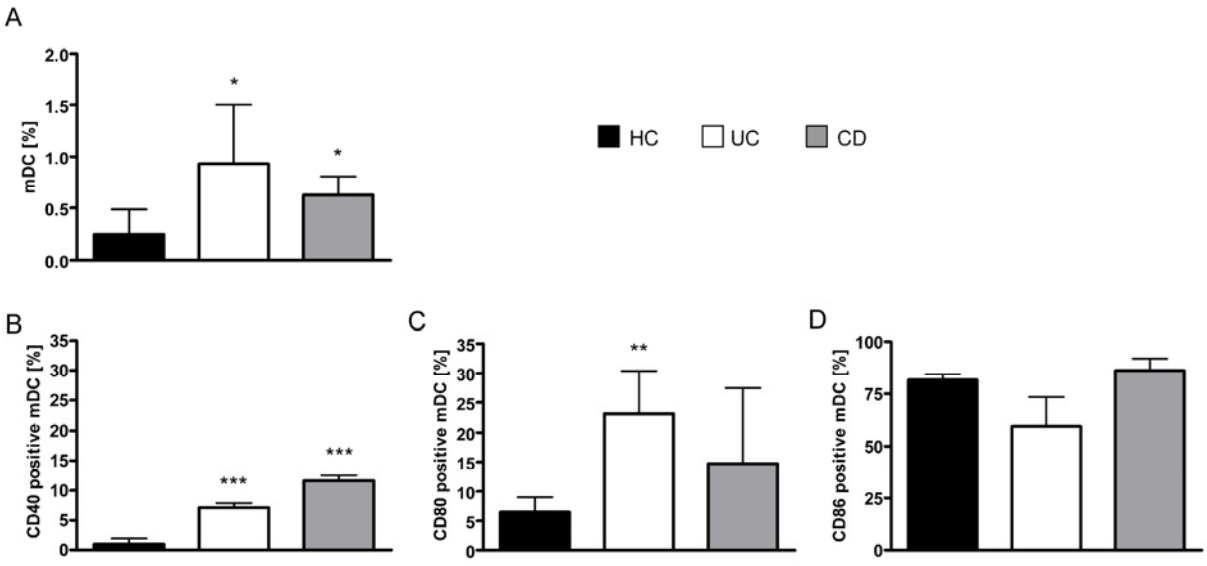

Figure 37: Increased frequency of dendritic cells in the mucosa of IBD patients. In patients with active IBD an increased frequency of (A) mDCs is found compared to controls. More mucosal mDCs from UC and CD patients display an activated phenotype with a significantly increased fraction of (B) CD40 and (C) CD80 expressing mDCs. (D) No significant difference in CD86 expressing mDCs could be observed. Bar graphs summarize data from 10 patients or controls within each group. HC – Healthy Control, UC – Ulcerative Colitis, CD – Crohn's Disease. Asteriks denote statistical significance $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

5 Discussion

DCs are powerful APCs of hemopoietic origin that are adept at both stimulating naïve TCs and also controlling the quality of subsequent immune responses. They are thought to play an important role in the maintenance of tolerance depending on their location and maturational state [13]. The transport of antigen from the site of infection to the draining LNs by DCs is a crucial component in this process. Besides contributing to adaptive resistance against microbial antigen, DCs have also been reported to be involved in chronic inflammation [162;163]. Due to their dichotomous response to antigenic, particularly microbial stimuli they are also involved in the control of peripheral as well as local immunity and tolerance [23]. Altogether, DCs are believed to take the center stage for the understanding of IBD [164;165]. Ultimately, mature and activated APCs induce and probably perpetuate an imbalance of effector TCs and regulatory TCs [166;167]. Indeed, various animal studies suggest their active involvement in the pathogenesis of IBD, but little is known about native, i.e. non-monocyte generated, human DCs and their handling of microbial antigens in IBD [168-172;173-176]. Our group focused on two distinct subsets of DCs characterized by their different expression levels which were isolated from human peripheral blood. The main aspect of this thesis lies in the investigation of human mDCs.

5.1 Human dendritic cells in the periphery and in secondary lymphatic organs

In humans, two peripheral blood DC subsets have been distinguished based on the expression of CD11c, an important adhesion molecule [177;178]. $CD11c^+$ DCs express myeloid-associated antigens and they are known to be immuno-stimulatory with a greater capacity for antigen uptake. On the other hand $CD11c^-$ DCs display features of the lymphoid lineage and require IL-3 for their development into pDCs [18;179]. They possess a lack of myeloid antigens and express high levels of CD123 (IL-3R) [180].

In this study suitable IBD patients which were off steroids, immunomodulators, immuno-suppressents and any biologics to prevent any influence of these drugs on DC function and their phenotype, were analyzed in comparison to healthy controls. Furthermore, patients with an infectious diarrhea (infectious colitis) were also analyzed as another site of inflammation in

the gut. Compared to other studies in which the authors either worked with monocyte derived DCs (MoDCs) or pooled several different myeloid DC subsets, this study is predicated on primary, highly purified DCs [181-183]. The focus in this work lies on mDCs ($CD1c^+$ $CD11c^+$ $CD14^-$ $CD19^-$) since previous work from our group demonstrated that their frequencies in IBD patients strikingly correlate with the disease activity and is sufficient for conducting functional studies [144].

It succeeded within the scope of this work to establish a certain method which allows us to isolate human blood mDCs with a high purity of at least 95%. Most notably DCs could be isolated from IBD patients with the same purity. The results obtained from isolating these cells corroborated previous findings in which a scarcity of circulating, immature peripheral blood DCs could be observed in patients with active IBD [144]. This finding strikingly correlates with the extent of inflammation. The major limitation of this study is the necessity to include different subjects into different experiments, as the number of purified DCs available from each individual donor sample is very limited and patients cannot be studied serially due to the form of medication and treatment they are undergoing.

5.1.1 Increased frequency of dendritic cells in the inflamed mucosa

It is known that IBD results from an inappropriate response of a defective mucosal immune system to the indigenous flora and luminal antigens. Since intestinal DCs play a key role in monitoring the intestinal lumen by continuously sampling and processing luminal antigen the location of inflammation was investigated [184;185]. In this work an increased frequency of mucosal mDCs in inflamed mucosal segments of patients with UC and CD was detected. The results presented in this work were always limited due to the availability of human tissue simply because IBD patients just undergo surgery in case of strong inflammation and side-effects. This finding confirms previous observations from studies in IBD animal models where the authors described subepithelial DC clusters and an accumulation in the lamina propria of the inflamed colonic mucosa [169-171]. Animal studies have a big advantage over human studies based on their indefinite bioavailabilty. Here, the authors could show an accumulation of mononuclear cells, i.e. DCs, in the lamina propria in the SCID mouse model of colitis [171;173;175]. Several studies on human IBD attempted to enumerate DCs by FACS or

immunohistochemistry in the human colonic mucosa. Some of them reported an increased frequency and correlation with the inflammatory activity. However, the results were mostly limited in UC [181;182;186;187]. Unfortunately, none of the groups could clearly identified DCs, since they either used CD83 or CD209 antibodies, which are both known to be expressed by a number of other leukocytes likewise such as monocytes, B-cells and lymphoblasts or cross react with monocytes, vascular and lymphoid endothelium, respectively. Another factor which exacerbates the process of identifying DCs in human tissue is their low number [21]. DCs represent less than 0.5% of peripheral blood mononuclear cells. Therefore, elaborate methods for the isolation of DCs from human tissue need to be established in future studies. Especially, the analysis of tissue taken from endoscopic surgeries need to be evaluated in future experiments, but the low frequency of DCs in tissues and the difficulty in obtaining large amounts of normal intestinal tissue makes such experiments very difficult to perform.

In addition to the increased frequency of mucosal mDCs in the inflamed mucosa of IBD patients, an increased number of mucosal mDCs that display an activated phenotype as indicated by the expression of the two co-stimulatory molecules CD40 and CD80 which are critical for TC activation were observed [149-151]. The greater number of CD40 expressing mucosal mDCs in CD and UC correlates with cultured circulating mDCs from these patients. This corroborated the expression of CD40 on mucosal DCs in CD which could be demonstrated by Hart et al. [188]. CD40-CD40L interactions have been identified to be important for the development and control of inflammation in several IBD animal models [169;172;189;190]. An up-regulated CD40L expression has been reported in mucosal lesions of human IBD and CD40 ligation is furthermore associated with an increased NFκB activation and secretion of inflammatory cytokines in human colonic fibroblasts [191-194]. It is known that DCs use PRRs to recognize and respond to microbial structures [33]. Therefore other groups investigated the expression of TLR2 and TLR4, two important receptors during the activation of TCs by DCs. Hart and colleagues could further demonstrate that cells from IBD tissue show increased numbers of TLR2-positive and TLR4-positive DCs [188]. That finding also indicates that DCs from IBD patients might be able to show an increased response to microbial excitation in the human gut.

Collectively, the above findings indicate on the one hand that there is a loss of human DCs in the periphery and on the other hand an accumulation of DCs at the center of inflammation in

the gut of IBD patients. The detailed process of migration will have to be dissected in future studies.

5.1.2 Dendritic cells from IBD patients display a different phenotype

In order to efficiently activate naïve TCs, DCs must be able to up-regulate their expression of both MHC and co-stimulatory molecules. This is achieved by following an exposure to various signals provided by inflammatory cytokines and pathogen derived products [13]. However, early events after peripheral DC activation are not well investigated. Since DCs play an important role at the modulation of the immune system it is relevant to further understand these cells and eventually be able to regulate the events of DC maturation in the future. Finally, that will open a way to control the final effector function of DCs.

In this study previous findings from our group in which we were able to demonstrate that patients with an acute illness of UC and CD experience a significant drop in their peripheral DC populations were extended by a direct correlation of their phenotypic key features to expression of important co-stimulatoy molecules and maturation markers [144]. The results obtained from culture experiments following staining for several important DC markers showed that a significantly greater number of cultured mDC in IBD express CD40, compared with healthy controls. Upon contact with LPS between 74% to 91% of mDCs from IBD patients express CD40. In CD the frequency of CD40 expressing mDCs is slightly higher in flaring patients than during RM. It could be observed that the addition of LPS as a stimulus is dose-dependent regarding CD40 expression and slight variances in the addition of LPS lead to slightly different CD40 expression patterns. Therefore, one stock of freshly prepared LPS was used for one set of experiments. That may explain why sometimes more mDCs express CD40 in future experiments and why the data doesn't show exactly the same trend. These findings correlate with a slightly higher number of CD40 expressing mucosal mDCs in IBD patients and may reflect the intrinsic difference in the regulation of TC activation during both conditions. The same trend was observed for the co-stimulatory CD80 molecule. In contrast, the phenotype of freshly isolated mDCs does not diverge from the phenotype of healthy controls. Since one would not expect activated DCs or the encounter of LPS in the peripheral circulation, other than in sepsis, as this would cause Paul Ehrlich's "horror autotoxicus", the

data corroborate with previous observations [144;195;196]. Taken together, the results presented in this work confirm that an exposure to LPS results in full activation of mDCs, as indicated by high levels of CD40 and CD80.

Collectively, the observed up-regulation of co-stimulatory molecules on mDCs after stimulation with LPS was expected and correspond well with the published expression of TLR4 by mDCs [197]. It has been suggested that $CD11c^+$ subsets represent an immunologically mature state of DCs or recirculating DCs [177]. Hence, DC activation is presumed to be a peripheral event. Due to inflammation or necrotic stimuli DCs in peripheral tissue experience activation and finally migrate to T lymphocyte-rich interfollicular regions where they finally present antigen to naïve TCs [198-200]. Accordingly, as expected, the above findings indicate that DCs from IBD patients show changes in their phenotype resulting in a constitutive activation of blood mDCs in CD and particularly in UC, even in clinically very mild IBD. This suggests that these cells are either recirculating from peripheral tissue or are being activated by systemic influences as a result of an advanced course of disease in the GI.

5.1.3 Disturbed cytokine balance in IBD

A crucial step leading to inflammation or autoimmunity, i.e. SLE, is an overproduction of specific cytokines and it is known that subsets of DCs can be a major source in this process [201]. The cells reside in the peripheral tissues during the immature state where they are endocytic and express low levels of MHC class II and co-stimulatory molecules. The latter could be confirmed within the work of this thesis. It has been suggested by others that "steady state" DCs are capable of maintaining tolerance by inducing regulatory TCs or the deletion of autoreactive TCs [202]. Following maturation, the cells lose their endocytic ability, up-regulate MHC class II as well as co-stimulatory molecules, and present antigen to TCs for the initiation of immune responses [13]. Finally, DCs influence TC proliferation and effector function by providing co-stimulation and establish a special cytokine environment at the time of TC priming [203-205]. During the active stage of disease, effector TCs (T_H1 and T_H2) predominate over regulatory TCs as a consequence of naïve TCs (T_H0) preferably differentiating into T_H1 cells [206]. In IBD this balance between regulatory and effector TCs is known to be disturbed. Especially pro-inflammatory cytokines play an important role in the pathogenesis of IBD since they support the events of inflammation in the gut. Increasing

interests demands in the use of pro-inflammatory cytokines as starting points for new therapies in acute IBD, i.e. the clinical efficacy of targeting TNF-α has to be mentioned as it clearly indicates that cytokines are one of the therapeutic targets of chronic inflammatory disorders such as IBD [207]. Moreover, Infliximab, a mouse/human chimeric mAb, which binds to TNF-α has shown efficacy in the treatment of moderate-to severe CD and a number of CD-related complications [208-210].

In addition, several other studies have also demonstrated that subsets of DCs play an important role in the stimulation and differentiation of T_H1 or T_H2 cells during immune responses [211-213]. Therefore, cytokine secretion of important pro- and anti-inflammatory cytokines was studied to further investigate whether DCs from IBD patients, respectively, secrete increased amounts of pro-inflammatory cytokines in comparison to healthy controls.

5.1.3.1 Myeloid dendritic cells from IBD patients secrete higher levels of pro-inflammatory cytokines

Altogether, human mDCs cultured without any stimulus secrete very low levels of IL-6, IL-8, and TNF-α. Further addition of LPS increases secretion of all observed cytokines which has been demonstrated by other groups as well [214;215].

TNF-α is a pro-inflammatory mediator that plays an integral role in the pathogenesis of IBD. In addition, mounting evidence indicates a genetic association between TNF-α and UC. Furthermore, increased TNF-α levels have been found in studies with IBD patients [214;216;217]. Moreover it is well-known that TNF-α is important for the migration of DC. The absence of TNF-α results in a different generation and maturation of DCs from macrophages. It could be verified that most notably immature DCs are sensitive to LPS and that they secret more TNF-α than mature cells [218]. Furthermore, studies of human mDCs displayed a significant TNF-α production after stimulation with LPS [214]. However, the authors did not differ between different types of diseases and possible changes in the function of mDCs. In this work it could be clearly demonstrated that mDCs from IBD patients secrete significantly greater amounts of the two prototypic IBD inflammatory cytokines TNF-α and IL-8 upon contact with LPS. These results underscore the observed phenotypic differences. An increased secretion of these two cytokines upon contact with microbial antigens likely encountered in the gut, such as LPS, attracts other leukocytes and may thereby contribute to the perpetuation of inflammation

known to occur in IBD [165]. The high secretion of TNF-α results in an accumulation of DCs in the gut of IBD patients due to boosted events of migration. This finally correlates with the demonstrated higher frequency of DCs in the inflamed tissue [219]. Previous findings have reported higher tissue levels of TNF-α in patients suffering from CD than in UC and that there is also a correlation in conjunction with the activity index of both diseases [216;217;220]. Additionally, it could be demonstrated that high concentrations of TNF-α result in an increased permeability of tight junctions [221]. Nikolaus et al. have shown that neutrophils from patients with intestinal inflammation secrete high levels of TNF-α and IL-1β in comparison with normal controls [215]. Since TNF-α is a key cytokine in RA and other systemic diseases as SLE it is not surprising that groups have found high levels of TNF-α in either MoDC and in serum [222;223]. IL-8 could be also detected by others in synovial fluid from patients with various inflammatory rheumatic diseases, and mucosal levels of IL-8 were elevated in patients with active UC [224;225].

There is also growing evidence that pro-inflammatory IL-6 plays a crucial part in the uncontrolled intestinal inflammatory process, which is a main characteristic of IBD [226]. Up-regulation of IL-6 production has been observed in a variety of chronic inflammatory and autoimmune disorders such as thyroiditis, type I diabetes, RA, systemic sclerosis, psoriasis and neoplasms [227-229]. The results in this work showed an increase in the secretion of IL-6 upon LPS stimulation. Moreover, mDCs of IBD patients secreted more IL-6 than their healthy counterpart. This observation is in line with previous studies where the authors worked with MoDC from patients with RA [222]. In patients suffering from SLE higher serum concentrations of IL-6 had been demonstrated as well [223]. However, UC patients during an acute stage of disease showed an unexpected reduction in the secretion of IL-6. The differences in the level of IL-6 are conducive to the disarranged TC polarization. To date, attention has focused on a novel subset of IL-17-producing $CD4^+$ TC termed T_H17 [230-232]. T_H17 cells are distinct from T_H1 and T_H2 and recent evidence suggest that TGF-β and IL-6 are important for the differentiation into T_H17 cells [233]. Furthermore, IL-6 production by APCs has recently been shown to render effector TC insensitive to the action of regulatory TCs and change the repertoire of antigens presented to DCs [234;235]. Hence, the observed high secretion of IL-6 by mDCs in IBD may overcome regulatory TC populations specific for commensal antigens. On the other hand, the observed reduction in the secretion of IL-6 during acute UC might be due to a possible defect in the T_H17 response.

All together, the data shows that mDCs from IBD patients are more sensitive to the encounter with pathogens, i.e. LPS. Patients secrete higher levels of pro-inflammatory cytokines indicating that their tolerogenic ability is defective. The results clearly show that peripheral DCs possess a modified reaction upon LPS stimulation. The investigated increase in the secretion of pro-inflammatory cytokines after stimulation with LPS mark an important aspect in the pathogenesis of IBD. Cytokines derived from DCs may have a direct local effect, regulating survival or function of mucosal populations, or they may act indirectly by redirecting TC differentiation. Most changes in the cytokine profile were noticed in UC. However, no distinct statement can be made if there is an ascendence towards $T_H1$or T_H2 in UC. In addition the data does not demonstrate clear evidence which underlies the published predominance of T_H1 cells in patients suffering from CD [96;236;237].

5.1.3.2 Myeloid dendritic cells from IBD patients secrete higher levels of anti-inflammatory IL-10

IL-10 is known as an immunoregulatory cytokine that is secreted by T_H2 subsets of T-helper cells and strongly downregulates the production of pro-inflammatory cytokines. Its regulatory role outside of IBD related studies are well established as it inhibits T_H1 cell activation and moreover downregulates the acute inflammatory response [238;239].

The results in this investigation showed that human mDCs from IBD patiente secrete significantly higher levels of IL-10 upon contact with LPS. However, the observed increase in the secretion of IL-10 alone is not sufficient to reflect a regulatory potential of mDCs and an attempt to balance the immune response towards microbial stimuli [23]. Future experiments, like studying immunosuprressive TGF-β, will be required to support this statement. Otherwise, the findings are in line with previous publications. It has been shown by others that circulating levels of IL-10 are increased within active disease in IBD [240]. Of note, an IL-10 knockout mouse has been reared that develops an enterocolitis which will be prevented by the administration of IL-10 [241-243]. This finding corroborates the action of IL-10 against chronic stimulation of the intestinal immune system. Consequently, despite increased circulating levels of IL-10, a relative tissue deficiency may be present in IBD that may contribute to the pathogenesis of the disease. However, DCs are known for their dual role in the polarization of immune responses.

5.1.4 Inflammatory response by myeloid dendritic cells of patients with IBD

The observed increased frequency of co-stimulatory molecule expressing mDCs and their increased expression of TLR in IBD patients may drive their maturation and suspected hyperactivation upon encountering intestinal microbial antigens. This suspected hypersensitivity to microbial antigens may also be related to their sampling or the amount of antigen uptake. Sophisticated DC antigen sampling mechanisms have recently described in *in vitro* and animal studies [185;244]. Since a distinct response of mDCs from IBD patients to LPS was observed, their uptake of fluorochrome labeled LPS was studied. Here, differences between IBD patients and healthy controls were found. While an increased LPS uptake can already be observed in mDCs from IBD in RM when cultured for an extended period of time, mDCs from flaring IBD patients take up substantially more LPS from the very beginning and increases continues over the entire culture period of 24 hrs.

The observed inflammatory response of mDCs and postulated migration to the gut may also occur in other intestinal inflammatory diseases, such as infectious colitis or diverticulitis. However, the finding that even mDCs from IBD patients in RM show a more pronounced inflammatory response to LPS than mDCs from healthy controls strongly argues against this notion and finally suggests an intrinsic defect of mDCs or a regulatory problem. This hypothesis is supported by the results of the LPS uptake experiments. Accordingly, differences between IBD patients and healthy as well as during RM and acute FU for the particular disease could be observed. Furthermore, this interpretation is also indirectly supported by genetic data, showing mutations in the TLR4 receptor in IBD patients [245]. Another group demonstrated that DCs from disease tissue show a higher expression of TLR2 and TLR4, which may contribute to altered microbial recognition [188]. Here, mDCs from controls and IBD patients were examined for their mRNA expression levels of TLR2, which interacts with peptidoglycan and lipoprotein from gram-neagtive bacteria, and TLR4, a receptor for LPS from gram-negative bacteria [246-248]. Consistent with previous findings that detected TLR2 on the majority and in some cases TLR4 in mDCs, we documented a significant higher TLR2 expression in UC patients and an increased expression of TLR4, which is most pronounced in acute FU [33;188;249]. However, TLR4 expression or TLR4 receptor polymorphisms do not fully explain the inflammatory LPS response by IBD mDCs in RM. The correlation between LPS uptake and inflammatory cytokine secretion in remission

suggests that mDCs from IBD patients may also use non-TLR4 mediated LPS signaling pathways which have been reported by other groups [250]. A generalized increase in TLR2 and TLR4 in the inflamed intestinal tissue has been previously reported in the DSS colitis mouse model [251]. In human IBD, an increase in the expression of TLR2 and TLR4 has been reported on epithelial cells, and 10-20% of cells in the lamina propria [252]. Thus, an overexpression of TLRs by DCs and other cells which interacts with the microbial flora may lead to an increase in the recognition of bacterial products and enhanced responses. However, relative mRNA expression levels by mDCs after stimulation with LPS show a decrease of TLR2 in CD whereas UC patients express similar levels as controls. The expression pattern of TLR4 shows a trend to be up-regulated in IBD patients, which is most significant in UC during FU and CD during RM. Unfortunately, UC patients in RM express less TLR4 compared to all other samples. One problem in the interpretation of this data lies in the minor amount of cells which was available as starting material to perform RT-PCR experiments. Therefore, it would be interesting to investigate the expression levels of TLRs in a time course. Unfortunately, that remains unsolved due to the low cell counts. However, in certain cases it was possible to observe an enhanced mRNA expression.

Taken together, the data demonstrate an inflammatory phenotype, preferred secretion of inflammatory cytokines and expression of co-stimulatory molecules required for TC activation by human mDCs in patients with IBD. This response may be due to an increased antigen uptake by activated mDCs from flaring IBD patients as the results from the LPS uptake study nicely suggest. The increased frequency of activated mDCs in the inflamed mucosa of IBD patients could indicate migration of DCs to the gut in active IBD.

5.2 *Saccharomyces boulardii* exhibits anti-inflammatory potential through modulation of dendritic cells

It has been demonstrated in clinical trials that the yeast *Sb* is efficient in inflammatory and infectious disorders of the gastrointestinal tract [119-124]. However, little is known about how the yeast unfolds its anti-inflammatory properties in human. Most experimental research from *in vitro* studies with cell cultures and animal models focused on the prevention of microbial pathogen adherence, translocation of the commensal microbial flora, investigation of neutralization of bacterial toxins (i.e. *Clostridium difficile* toxin A or Cholera toxin), toxin

related signaling, maintenance of normal intestinal permeability and barrier function as well as control of epithelial electrolyte transport and luminal secretion [138;253-264]. However, a positive outcome in clinical pilot studies in IBD patients as well as beneficial effects of *Sb* in immunological (adoptive transfer model in SCID mice) and chemical (DSS colitis) animal models of IBD imply additional, directly anti-inflammatory properties of *Sb* [129-131;265;266]. Further, two of these studies suggested that the anti-inflammatory properties of *Sb* may be mediated through an inhibition of pro-inflammatory lymphocyte migration towards MLNs and moreover an inhibition of pro-inflammatory polarization of $CD4^+$ TCs [265;266]. However, the authors have not studied this effect in the human system.

In this part the effects of *Sb*S on human mDCs and their control of TC activation were investigated. Since experiments with a *Sb*S dilution of 1:2 induced an extensive cell death this approach was not further investigated.

5.2.1 Administration of *Saccharomyces boulardii* culture supernatant results in changes of dendritic cell function

The expression of co-stimulatory molecules on APCs is an important pre-requisite for TC activation [267]. Their up-regulation and the additional expression of activation molecules on the mDC surface mark not only a phenotypic, but dramatic functional change from being inducers of peripheral tolerance to potent activators of immune effector cells, such as TCs [23;196]. Therefore, this process was investigated by assessing the expression of typical activation markers, co-stimulatory molecules as well as maturation markers on LPS stimulated mDCs in the presence or absence of different dilutions of *Sb*S. As mentioned earlier, microbial antigen surrogate motif and model TLR ligand LPS was used to stimulate mDCs as this mimics the situation these cells may encounter in the gut. Consistent with the literature and the results presented above, freshly isolated, immature and inactive mDCs expressed virtually no detectable numbers of the three markers [195]. Further addition of LPS induced the expected maturation of mDCs as indicated by their increased expression of these molecules. However, when cultured in the presence of LPS and *Sb*S, substantially fewer mDCs express CD40 and CD80 and CCR7. The reduced expression of CD197 by *Sb*S in mDCs which was observed in this work, may prevent DC migration from the peripheral circulation to the sites of inflammation known to occur in chronic inflammatory conditions such as IBD [144].

Taken together, the presence of *Sb*S modulates mDCs and results in significant phenotypic changes with a potential to prevent migration of these cells which needs to be investigated in future studies.

5.2.2 Administration of *Saccharomyces boulardii* culture supernatant results in changes of dendritic cell immune responses

Besides *Sb*S's ability to reduce the number of activated mDCs, the yeast moreover modulates mDCs to secrete less TNF-α and IL-6 upon contact with LPS compared with LPS only stimulated mDCs. These data underscore the observed phenotypic changes discussed above. An increased secretion of TNF-α and IL-6 upon contact with microbial antigens encountered in the gut attracts other leucocytes and may thereby contribute to the perpetuation of inflammation known to occur in IBD [61;71]. However, DCs are also known for their dual role in the polarization of immune responses. Surprisingly, *Sb*S was not only able to reduce the secretion of pro-inflammatory cytokines but also able to increase the secretion of anti-inflammatory IL-10 to further boost the natural regulatory potential of mDCs [23]. It is not possible to rule out that *Sb*S induced a down-regulation of co-stimulatory molecules on LPS stimulated mDCs is in part mediated through direct effects of *Sb*S on LPS. Animal studies identified a protein phosphatase in the probiotic yeast that had a greater ability to dephosphorylate LPS of *E. coli*, which when injected in rats produced substantially less TNF-α and no organic lesions compared with the non *Sb* exposed LPS [268]. However, this observation cannot explain the further up-regulation of IL-10 which is also increased by LPS alone. Additional evidence for a modulation of the immune response by *Sb* which may also help to explain its clinical benefit in inflammatory and infectious conditions comes from *in vitro* and animal studies. Orally administered *Sb* was shown to increase the production of secretory IgA and the secretory component of immunoglobulins in growing rats and monoassociated germ free mice thereby augmenting the hosts first line of defense of the innate immune system in the gut [269;270]. Furthermore it has been shown that the yeast blocks NF-κB activation, IL-8 and TNF-α gene expression, IL-8 production, and secretion by lymphoid and non-lymphoid cells [263;271-273].

5.2.3 *Saccharomyces boulardii* culture supernatant induces phenotypic and functional changes in myeloid dendritic cells from IBD and IC patients

Having shown that administration of *Sb*S results in changes of DC phenotype and function in mDCs from healthy controls, we aimed to verify this effect in IBD and IC patients.

We were able to reproduce effects of the yeast on LPS stimulated mDCs from IBD patients. The number of CD40, CD80 and CCR7 expresssing mDCs was significantly reduced in the presence of *Sb*S and LPS compared to LPS alone. Moreover, the second control group consisting of patients suffering from an infectious colitis showed the same trend as well.

Furthermore, *Sb*S modulates mDCs isolated from UC and CD patients to secrete less IL-6 and TNF-α upon LPS stimulation. This is in line with previous findings by other groups. In previous studies the authors were able to demonstrate a reduction in the production of pro-inflammatory cytokines and a simultaneous boost in the intestinal production of anti-inflammatory cytokines like IL-10 [274].

Unfortunately, we were not able to confirm the increase in the secretion of IL-10 for the overall IBD population. We observed a slightly higher IL-10 secretion in *Sb*S exposed mDCs isolated from CD already in remission, which did not further increase during flare-up. This suggests distinct effects on regulatory pathways in CD vs. UC and emphasized their pathophysiological differences. Another group was able to demonstrate in rats that the administration of *Sb* leads to an increase in the production of TGF-β another anti-inflammatroy cytokine which has been shown to be a key cytokine in the inflamed tissue of patients with [275;276]. It could be confirmed within the work of this thesis that patients with CD secrete more or similar levels of IL-10 when *Sb*S was added to LPS stimulated mDCs. It would be interesting to investigate the production of TGF-β as well in future experiments.

In summary, we were able to reproduce many of the anti-inflammatory effects of *Sb*S observed in healthy control mDCs also in IBD patients. We noted differences between the two entities and disease activity levels. Cytokine levels were not affected in IC patients, implementing a distinct modulation of IBD mDCs. However, additional studies are required to detail the specific mechanisms of the observed differences.

5.2.4 Effective stimulation of naïve T cells by myeloid dendritic cells is suppressed by *Saccharomyces boulardii* culture supernatant

DCs are uniquely able to activate naïve $CD4^+$ TCs [277;278]. In rats under steady state condition around 800,000 DCs migrate each day from the small intestine to the MLNs [279]. These migrating DCs carry self-antigens and are all CCR7-positive, which is in line with the findings described above [279;280]. Finally, these DCs are required for the maintenance of oral tolerance [281]. Rimoldi et al. have described a mechanism by which migrating DCs may mediate tolerance to intestinal antigens [282]. The authors showed that human blood DCs cultured with thymic stromal derived lymphopoietin are unable to stimulate the production of inflammatory cytokines and IFN-γ in an alloreaction with TCs.

To further investigate DC properties, the ability of blood mDCs from healthy controls was tested to induce proliferation from $CD4^+$ TCs. It could be demonstrated that mDCs are able to active naïve TCs. Further addition of LPS as a stimulus to the MLR did not show any significant differences in the level of TC proliferation. Furthermore, when naïve TCs were mixed with purified mDCs higher concentrations of IFN-γ, but only moderate levels of IL-4 were measured by intracellular staining. These results indicate that the mDCs induced an inflammatory T_H1 phenotype in the responding TC. This is consistent with previous findings were the authors compared different steady-state intestinal lymph DCs and their potential to activate naïve TCs [283]. This might be a good approach for future studies in which the phenotype of TC from IBD patients should be further investigated. It is already widely accepted that TCs of patients suffering from CD preferably differentiate into T_H1 cells [206]. Generally, more studies are needed to further clarify this subject.

The effects of *Sb*S on mDC were not restricted to phenotypic changes, but corroborated by functional consequences as well. It could be demonstrated within the work of the thesis that *Sb*S is able to suppress mDC mediated TC activation in an allogenic MLR. This dose-dependent effect may be exploited in the management of enteric disorders such as IBD, which is believed to result from an aberrant response of DC to microbial antigens.

5.2.5 The active component of *Saccharomyces boulardii* culture supernatant

The described phenotypical and functional changes by *Sb*S raised the question which component of the yeast might be responsible for the investigated effects. Thus, yeast supernatant was fractionated by membrane partition chromatography till a maximum weight limit of 3 kDa.

The previous observed decrease in the number of CD40, CD80 and CCR7 expressing mDCs in after stimulation and in the presence of *Sb*S was also noticed for all permeates down to 3 kDa. Furthermore, the investigation of other maturation markers like CD83 and CD86 displayed no differences at all. Likewise results were monitored in the secretion of key pro-inflammatory as well as anti-inflammatory cytokines. The secretion of IL-6 and TNF-α showed a decrease for all permeates whereas an increase in IL-10 secretion was observed. This confirms the results discussed above. To further corroborate this data, the retentates of the ultrafiltration process were used in identical experiments and not surprisingly the opposite results were found. The retentates did not induce the phenotypic changes and cytokine secretion pattern seen with the *Sb*S permeates. This suggests, that the active component is included in all permeates. In summary, since all permeates down to <3 kDa resulted in a similar phenotype and cytokine secretion compared with non-ultrafiltrated, the active component in *Sb*S appears to have a molecular weight smaller than 3 kDa.

Taken together, the presence of *Sb*S modulates mDCs and results in significant phenotypic changes with potential to prevent migration of these cells. More research is required to describe the molecular structure of the active constituent in *Sb*S. The data suggests that the probiotic yeast *Sb* may exhibit its anti-inflammatory potential through modulation of DC phenotype, function and migration by inhibition of their immune response to bacterial microbial surrogate antigens such as LPS. It cannot be guaranteed that baker's yeast, which is genetically related to *Sb* may have similar properties but this context was not observed within this work as the goal was to explore its potential mechanisms with regard to inflammation. This represents an important topic for future experiments. Since the clinical relevance and pertinence of the study is limited future studies need also to investigate a beneficial effect of *Sb* in IBD. Therefore, further studies with samples from IBD patients would be required to provide evidence for an efficacy in IBD. However, such studies will require a substantial amount of time, since only carefully selected patients off steroids, immunomodulators and

biologics could be included in order to avoid any influence of such drugs on the experimental outcome and eventually the demonstrated decrease in the frequency of DCs in IBD patients.

5.3 Perspective

The results obtained in this study support the notion that DCs play an important role in patients with IBD. They are important for the understanding of how certain events take place during inflammation. It could be shown that mDCs from IBD patients respond different to various stimuli compared to healthy individuals. This yields to a modified and disrupted inflammation process during IBD. The physiological relevance of intestinal accumulation by DCs is illustrated by the finding that frequency of mDCs is higher in the inflamed tissue compared to healthy controls. It will be interesting in future studies to further investigate the basis of the observed effect, particular the activity at the place of inflammation itself. Therefore it is important to establish a method which allows the isolation of DCs from mucosa and especially from specimen taken from endoscopic surgeries. Less material will be required and more important DCs will come directly from the site of inflammation, the gut. This process will be complicated by the fact that the overall occurrence of DCs is very low (0.1-0.5%) and that endoscopic specimen are very little [21;284]. Another important issue which needs to be addressed in future studies is the event of migration. Therefore, possible observations might be the study of important migration markers in the gut to gather information about the state of activation of the cells in the mucosa. Furthermore, direct exposure of DCs in the human gut should result in more information in the localization of the cells in the tissues itself which finally would offer valuable clues to ways the inflammation offense the tissues. Liu and colleagues nicely demonstrated in mice that DC development progresses from the macrophage and DC precursor to common DC precursors that give rise to pDCs and classical spleen DCs (cDCs), but not monocytes, and finally to committed precursors of cDCs (pre-cDCs). Eventually, pre-cDCs will enter LNs and migrate along HEVs and later disperse and integrate into the DC network [27]. This raises the question if there are any differences in human and primarily if there are checkpoints in DC development in patients suffering from chronic or autoimmune diseases.

Regarding the observed changes in the cytokine profile of IBD patients further studies would be necessary. Especially, the increasing interest in the role of two major TC specific cytokines, IL-17 and IL-23, which previously have been demonstrated to be very important needs to be investigated [226;285]. Several studies indentified IL-23 as an essential mediator of intestinal inflammation [286-289]. The authors show that IL-23 orchestrate an inflammatory cytokine cascade involving increased levels of IFN-γ, IL-6, IL-17, and TNF-α in the intestine.

Moreover, experiments on models with brain and joint inflammation indicated similar results [290;291]. Altogether, IL-23 is believed to be an important conductor of an inflammatory response which needs to be investigated for its role in IBD.

With the results presented in this thesis it would be very interesting to further study the effects of the probiotic yeast *Sb*. It could be shown that this yeast together with the according supernatant has effects on the regulation of co-stimulatory molecules in response to LPS and that the secretion of key cytokines is affected. However, various important issues remain unsolved. Additional experiments are required to support the hypothesis. Thus, it would be important to examine the effect of culture supernatants of other types if yeast without any known anti-inflammatory activity. Alternatively, other related microorganisms should be tested in this project. It would be also very interesting to further characterize the part of the yeast which is responsible for the observed effects. This can be addressed by testing the activity after dialyzing the supernatant against culture medium with 0.1 to 3 kDa cutoff membranes before adding to the mDC culture or moreover, by using the (1→3)-β-D-glucan isolated from *Sb* and other assimilable yeasts [292;293]. Since IL-12p70 and IL-23 capture a key role in IBD and it is known that they are secreted by activated DCs the analysis of these two cytokines might be important to confirm that *Sb* has an effect on human DCs.

Taken together, the data presented here shows only a fraction of possible roles of DCs during IBD. Future research is required to provide further insights into the procedures of DCs especially in human tissue and MLNs. These experiments will gain more insight into the complex procedures during IBD and will hopefully be successful in the fight against these diseases.

References

1. Mackay IR. Autoimmunity and primary biliary cirrhosis. Baillieres Best.Pract.Res.Clin.Gastroenterol. 2000;14:519-533.

2. Kimbrell DA, Beutler B. The evolution and genetics of innate immunity. Nat.Rev.Genet. 2001;2:256-267.

3. Medzhitov R, Janeway C, Jr. Innate immunity. N.Engl.J.Med. 2000;343:338-344.

4. Chaplin DD. 1. Overview of the human immune response. J.Allergy Clin.Immunol. 2006;117:S430-S435.

5. Heyzer-Williams LJ, Malherbe LP, Heyzer-Williams MG. Helper T cell-regulated B cell immunity. Curr.Top.Microbiol.Immunol. 2006;311:59-83.

6. Heyzer-Williams MG. B cells as effectors. Curr.Opin.Immunol. 2003;15:354-361.

7. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. Cell 2000;101:455-458.

8. Dempsey PW, Vaidya SA, Cheng G. The art of war: Innate and adaptive immune responses. Cell Mol.Life Sci. 2003;60:2604-2621.

9. Heyzer-Williams LJ, Driver DJ, Heyzer-Williams MG. Germinal center reaction. Curr.Opin.Hematol. 2001;8:52-59.

10. Medzhitov R. Toll-like receptors and innate immunity. Nat.Rev.Immunol. 2001;1:135-145.

11. Langerhans, P. Über die Nerven der menschlichen Haut. 44(44), 325. 1868. Virchows Arch Pathol Anat Physiol Klin Med.

12. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J.Exp.Med. 1973;137:1142-1162.

13. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245-252.

14. Barclay AN, Mayrhofer G. Bone marrow origin of Ia-positive cells in the medulla rat thymus. J.Exp.Med. 1981;153:1666-1671.

15. Katz SI, Tamaki K, Sachs DH. Epidermal Langerhans cells are derived from cells originating in bone marrow. Nature 1979;282:324-326.

16. Katz SI, Cooper KD, Iijima M, Tsuchida T. The role of Langerhans cells in antigen presentation. J.Invest Dermatol. 1985;85:96s-98s.

17. Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. Nat.Immunol. 2004;5:1219-1226.

18. Liu YJ, Kanzler H, Soumelis V, Gilliet M. Dendritic cell lineage, plasticity and cross-regulation. Nat.Immunol. 2001;2:585-589.

19. Reis e Sousa. Dendritic cells in a mature age. Nat.Rev.Immunol. 2006;6:476-483.

20. Rossi M, Young JW. Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. J.Immunol. 2005;175:1373-1381.

21. Van Voorhis WC, Hair LS, Steinman RM, Kaplan G. Human dendritic cells. Enrichment and characterization from peripheral blood. J.Exp.Med. 1982;155:1172-1187.

22. Romani N, Gruner S, Brang D et al. Proliferating dendritic cell progenitors in human blood. J.Exp.Med. 1994;180:83-93.

23. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu.Rev.Immunol. 2003;21:685-711.

24. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell 2001;106:259-262.

25. Grouard G, Rissoan MC, Filgueira L et al. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. J.Exp.Med. 1997;185:1101-1111.

26. Banchereau J, Briere F, Caux C et al. Immunobiology of dendritic cells. Annu.Rev.Immunol. 2000;18:767-811.

27. Liu K, Victora GD, Schwickert TA et al. In vivo analysis of dendritic cell development and homeostasis. Science 2009;324:392-397.

28. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat.Immunol. 2004;5:987-995.

29. Apostolopoulos V, McKenzie IF. Role of the mannose receptor in the immune response. Curr.Mol.Med. 2001;1:469-474.

30. Bajtay Z, Csomor E, Sandor N, Erdei A. Expression and role of Fc- and complement-receptors on human dendritic cells. Immunol.Lett. 2006;104:46-52.

31. Bryant P, Ploegh H. Class II MHC peptide loading by the professionals. Curr.Opin.Immunol. 2004;16:96-102.

32. Jarrossay D, Napolitani G, Colonna M, Sallusto F, Lanzavecchia A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur.J.Immunol. 2001;31:3388-3393.

33. Kadowaki N, Ho S, Antonenko S et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J.Exp.Med. 2001;194:863-869.

34. Cella M, Jarrossay D, Facchetti F et al. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat.Med. 1999;5:919-923.

35. Res PC, Couwenberg F, Vyth-Dreese FA, Spits H. Expression of pTalpha mRNA in a committed dendritic cell precursor in the human thymus. Blood 1999;94:2647-2657.

36. Siegal FP, Kadowaki N, Shodell M et al. The nature of the principal type 1 interferon-producing cells in human blood. Science 1999;284:1835-1837.

37. Summers KL, Hock BD, McKenzie JL, Hart DN. Phenotypic characterization of five dendritic cell subsets in human tonsils. Am.J.Pathol. 2001;159:285-295.

38. Lebre MC, Antons JC, Kalinski P et al. Double-stranded RNA-exposed human keratinocytes promote Th1 responses by inducing a Type-1 polarized phenotype in dendritic cells: role of keratinocyte-derived tumor necrosis factor alpha, type I interferons, and interleukin-18. J.Invest Dermatol. 2003;120:990-997.

39. Bacchetta R, Gregori S, Roncarolo MG. CD4+ regulatory T cells: mechanisms of induction and effector function. Autoimmun.Rev. 2005;4:491-496.

40. O'Sullivan B, Thomas R. CD40 and dendritic cell function. Crit Rev.Immunol. 2003;23:83-107.

41. Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. Science 1996;272:50-53.

42. Dilioglou S, Cruse JM, Lewis RE. Function of CD80 and CD86 on monocyte- and stem cell-derived dendritic cells. Exp.Mol.Pathol. 2003;75:217-227.

43. Appleman LJ, Berezovskaya A, Grass I, Boussiotis VA. CD28 costimulation mediates T cell expansion via IL-2-independent and IL-2-dependent regulation of cell cycle progression. J.Immunol. 2000;164:144-151.

44. Moser M, Murphy KM. Dendritic cell regulation of TH1-TH2 development. Nat.Immunol. 2000;1:199-205.

45. Ohshima Y, Delespesse G. T cell-derived IL-4 and dendritic cell-derived IL-12 regulate the lymphokine-producing phenotype of alloantigen-primed naive human CD4 T cells. J.Immunol. 1997;158:629-636.

46. Wenner CA, Guler ML, Macatonia SE, O'Garra A, Murphy KM. Roles of IFN-gamma and IFN-alpha in IL-12-induced T helper cell-1 development. J.Immunol. 1996;156:1442-1447.

47. Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. J.Immunol. 2005;174:4465-4469.

48. Lu J, Giuntoli RL, Omiya R et al. Interleukin 15 promotes antigen-independent in vitro expansion and long-term survival of antitumor cytotoxic T lymphocytes. Clin.Cancer Res. 2002;8:3877-3884.

49. Kelsall B. Recent progress in understanding the phenotype and function of intestinal dendritic cells and macrophages. Mucosal.Immunol. 2008;1:460-469.

50. Mowat AM, Millington OR, Chirdo FG. Anatomical and cellular basis of immunity and tolerance in the intestine. J.Pediatr.Gastroenterol.Nutr. 2004;39 Suppl 3:S723-S724.

51. Wittig BM, Zeitz M. The gut as an organ of immunology. Int.J.Colorectal Dis. 2003;18:181-187.

52. Berg RD. The indigenous gastrointestinal microflora. Trends Microbiol. 1996;4:430-435.

53. Hughes JB, Hellmann JJ, Ricketts TH, Bohannan BJ. Counting the uncountable: statistical approaches to estimating microbial diversity. Appl.Environ.Microbiol. 2001;67:4399-4406.

54. Hooper LV, Bry L, Falk PG, Gordon JI. Host-microbial symbiosis in the mammalian intestine: exploring an internal ecosystem. Bioessays 1998;20:336-343.

55. Madara JL, Parkos C, Colgan S et al. The movement of solutes and cells across tight junctions. Ann.N.Y.Acad.Sci. 1992;664:47-60.

56. Faria AM, Weiner HL. Oral tolerance: mechanisms and therapeutic applications. Adv.Immunol. 1999;73:153-264.

57. Harrison LC, Hafler DA. Antigen-specific therapy for autoimmune disease. Curr.Opin.Immunol. 2000;12:704-711.

58. Viney JL, Mowat AM, O'Malley JM, Williamson E, Fanger NA. Expanding dendritic cells in vivo enhances the induction of oral tolerance. J.Immunol. 1998;160:5815-5825.

59. Weiner HL. The mucosal milieu creates tolerogenic dendritic cells and T(R)1 and T(H)3 regulatory cells. Nat.Immunol. 2001;2:671-672.

60. Neurath MF, Finotto S, Glimcher LH. The role of Th1/Th2 polarization in mucosal immunity. Nat.Med. 2002;8:567-573.

61. Baumgart DC, Carding SR. Inflammatory bowel disease: cause and immunobiology. Lancet 2007;369:1627-1640.

62. Blumberg RS, Strober W. Prospects for research in inflammatory bowel disease. JAMA 2001;285:643-647.

63. Autschbach F, Braunstein J, Helmke B et al. In situ expression of interleukin-10 in noninflamed human gut and in inflammatory bowel disease. Am.J.Pathol. 1998;153:121-130.

64. Braunstein J, Qiao L, Autschbach F, Schurmann G, Meuer S. T cells of the human intestinal lamina propria are high producers of interleukin-10. Gut 1997;41:215-220.

65. Strobel S, Mowat AM. Immune responses to dietary antigens: oral tolerance. Immunol.Today 1998;19:173-181.

66. Weiner HL. Oral tolerance: immune mechanisms and treatment of autoimmune diseases. Immunol.Today 1997;18:335-343.

67. Marth T, Ring S, Schulte D et al. Antigen-induced mucosal T cell activation is followed by Th1 T cell suppression in continuously fed ovalbumin TCR-transgenic mice. Eur.J.Immunol. 2000;30:3478-3486.

68. Dalton DK, Haynes L, Chu CQ, Swain SL, Wittmer S. Interferon gamma eliminates responding CD4 T cells during mycobacterial infection by inducing apoptosis of activated CD4 T cells. J.Exp.Med. 2000;192:117-122.

69. Kraus TA, Toy L, Chan L, Childs J, Mayer L. Failure to induce oral tolerance to a soluble protein in patients with inflammatory bowel disease. Gastroenterology 2004;126:1771-1778.

70. Abraham C, Cho JH. Inflammatory bowel disease. N.Engl.J.Med. 2009;361:2066-2078.

71. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007;448:427-434.

72. WILHELM FABRY (1560-1624)--THE OTHER FABRICIUS. JAMA 1964;190:933.

73. Crohn BB, Ginzburg L, Oppenheimer GD. Landmark article Oct 15, 1932. Regional ileitis. A pathological and clinical entity. By Burril B. Crohn, Leon Ginzburg, and Gordon D. Oppenheimer. JAMA 1984;251:73-79.

74. Farmer RG, Hawk WA, Turnbull RB, Jr. Clinical patterns in Crohn's disease: a statistical study of 615 cases. Gastroenterology 1975;68:627-635.

75. Wilks S. Morbid appearances in the intestine of Miss Bankes. London Medical Times & Gazette 1859;2:264.

76. Rao SS, Holdsworth CD, Read NW. Symptoms and stool patterns in patients with ulcerative colitis. Gut 1988;29:342-345.

77. Meucci G, Vecchi M, Astegiano M et al. The natural history of ulcerative proctitis: a multicenter, retrospective study. Gruppo di Studio per le Malattie Infiammatorie Intestinali (GSMII). Am.J.Gastroenterol. 2000;95:469-473.

78. Loftus EV, Jr. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. Gastroenterology 2004;126:1504-1517.

79. Halfvarson J, Bodin L, Tysk C, Lindberg E, Jarnerot G. Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. Gastroenterology 2003;124:1767-1773.

80. Barrett JC, Hansoul S, Nicolae DL et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat.Genet. 2008;40:955-962.

81. Cho JH, Weaver CT. The genetics of inflammatory bowel disease. Gastroenterology 2007;133:1327-1339.

82. Duerr RH, Taylor KD, Brant SR et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 2006;314:1461-1463.

83. Rioux JD, Xavier RJ, Taylor KD et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat.Genet. 2007;39:596-604.

84. Hugot JP. Genetic origin of IBD. Inflamm.Bowel.Dis. 2004;10 Suppl 1:S11-S15.

85. Monsen U. Inflammatory bowel disease. An epidemiological and genetic study. Acta Chir Scand.Suppl 1990;559:1-42.

86. Orholm M, Binder V, Sorensen TI, Rasmussen LP, Kyvik KO. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. Scand.J.Gastroenterol. 2000;35:1075-1081.

87. Tysk C, Lindberg E, Jarnerot G, Floderus-Myrhed B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. Gut 1988;29:990-996.

88. Hampe J, Cuthbert A, Croucher PJ et al. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. Lancet 2001;357:1925-1928.

89. Hugot JP, Chamaillard M, Zouali H et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599-603.

90. Bouma G, Xia B, Crusius JB et al. Distribution of four polymorphisms in the tumour necrosis factor (TNF) genes in patients with inflammatory bowel disease (IBD). Clin.Exp.Immunol. 1996;103:391-396.

91. Gleeson MH, Walker JS, Wentzel J, Chapman JA, Harris R. Human leucocyte antigens in Crohn's disease and ulcerative colitis. Gut 1972;13:438-440.

92. Nakajima A, Matsuhashi N, Kodama T et al. HLA-linked susceptibility and resistance genes in Crohn's disease. Gastroenterology 1995;109:1462-1467.

93. Toyoda H, Wang SJ, Yang HY et al. Distinct associations of HLA class II genes with inflammatory bowel disease. Gastroenterology 1993;104:741-748.

94. van den Berg-Loonen EM, kker-Saeys BJ, Meuwissen SG, Nijenhuis LE, Engelfriet CP. Histocompatibility antigens and other genetic markers in ankylosing spondylitis and inflammatory bowel diseases. J.Immunogenet. 1977;4:167-175.

95. Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 1998;115:182-205.

96. Fuss IJ, Neurath M, Boirivant M et al. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J.Immunol. 1996;157:1261-1270.

97. Monteleone G, Biancone L, Marasco R et al. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. Gastroenterology 1997;112:1169-1178.

98. Duchmann R, Kaiser I, Hermann E et al. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin.Exp.Immunol. 1995;102:448-455.

99. Probert CS, Chott A, Turner JR et al. Persistent clonal expansions of peripheral blood CD4+ lymphocytes in chronic inflammatory bowel disease. J.Immunol. 1996;157:3183-3191.

100. Soderholm JD, Peterson KH, Olaison G et al. Epithelial permeability to proteins in the noninflamed ileum of Crohn's disease? Gastroenterology 1999;117:65-72.

101. Holzapfel WH, Haberer P, Snel J, Schillinger U, Huis i, V. Overview of gut flora and probiotics. Int.J.Food Microbiol. 1998;41:85-101.

102. Gorbach SL. Lactic acid bacteria and human health. Ann.Med. 1990;22:37-41.

103. Linskens RK, Huijsdens XW, Savelkoul PH, Vandenbroucke-Grauls CM, Meuwissen SG. The bacterial flora in inflammatory bowel disease: current insights in pathogenesis and the influence of antibiotics and probiotics. Scand.J.Gastroenterol.Suppl 200129-40.

104. Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. Science 2001;292:1115-1118.

105. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nat.Rev.Immunol. 2004;4:478-485.

106. Campieri M, Gionchetti P. Probiotics in inflammatory bowel disease: new insight to pathogenesis or a possible therapeutic alternative? Gastroenterology 1999;116:1246-1249.

107. Shanahan F. Turbo probiotics for IBD. Gastroenterology 2001;120:1297-1298.

108. Shanahan F. Probiotics in inflamatory bowel disease. Gut 2001;48:609.

109. Bibiloni R, Fedorak RN, Tannock GW et al. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. Am.J.Gastroenterol. 2005;100:1539-1546.

110. Gionchetti P, Rizzello F, Venturi A et al. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. Gastroenterology 2000;119:305-309.

111. Gionchetti P, Rizzello F, Helwig U et al. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. Gastroenterology 2003;124:1202-1209.

112. Rembacken BJ, Snelling AM, Hawkey PM, Chalmers DM, Axon AT. Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomised trial. Lancet 1999;354:635-639.

113. Marteau P, Lemann M, Seksik P et al. Ineffectiveness of Lactobacillus johnsonii LA1 for prophylaxis of postoperative recurrence in Crohn's disease: a randomised, double blind, placebo controlled GETAID trial. Gut 2006;55:842-847.

114. Prantera C, Scribano ML, Falasco G, Andreoli A, Luzi C. Ineffectiveness of probiotics in preventing recurrence after curative resection for Crohn's disease: a randomised controlled trial with Lactobacillus GG. Gut 2002;51:405-409.

115. Dieleman LA, Goerres MS, Arends A et al. Lactobacillus GG prevents recurrence of colitis in HLA-B27 transgenic rats after antibiotic treatment. Gut 2003;52:370-376.

116. Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN. Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology 1999;116:1107-1114.

117. Rachmilewitz D, Karmeli F, Shteingart S et al. Immunostimulatory oligonucleotides inhibit colonic proinflammatory cytokine production in ulcerative colitis. Inflamm.Bowel.Dis. 2006;12:339-345.

118. Waidmann M, Bechtold O, Frick JS et al. Bacteroides vulgatus protects against Escherichia coli-induced colitis in gnotobiotic interleukin-2-deficient mice. Gastroenterology 2003;125:162-177.

119. Attar A, Flourie B, Rambaud JC et al. Antibiotic efficacy in small intestinal bacterial overgrowth-related chronic diarrhea: a crossover, randomized trial. Gastroenterology 1999;117:794-797.

120. Can M, Besirbellioglu BA, Avci IY, Beker CM, Pahsa A. Prophylactic Saccharomyces boulardii in the prevention of antibiotic-associated diarrhea: a prospective study. Med.Sci.Monit. 2006;12:I19-I22.

121. Kotowska M, Albrecht P, Szajewska H. Saccharomyces boulardii in the prevention of antibiotic-associated diarrhoea in children: a randomized double-blind placebo-controlled trial. Aliment.Pharmacol.Ther. 2005;21:583-590.

122. McFarland LV, Surawicz CM, Greenberg RN et al. Prevention of beta-lactam-associated diarrhea by Saccharomyces boulardii compared with placebo. Am.J.Gastroenterol. 1995;90:439-448.

123. Sazawal S, Hiremath G, Dhingra U et al. Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials. Lancet Infect.Dis. 2006;6:374-382.

124. Villarruel G, Rubio DM, Lopez F et al. Saccharomyces boulardii in acute childhood diarrhoea: a randomized, placebo-controlled study. Acta Paediatr. 2007;96:538-541.

125. Edwards-Ingram L, Gitsham P, Burton N et al. Genotypic and physiological characterization of Saccharomyces boulardii, the probiotic strain of Saccharomyces cerevisiae. Appl.Environ.Microbiol. 2007;73:2458-2467.

126. Mitterdorfer G, Mayer HK, Kneifel W, Viernstein H. Clustering of Saccharomyces boulardii strains within the species S. cerevisiae using molecular typing techniques. J.Appl.Microbiol. 2002;93:521-530.

127. van der Aa KA, Jespersen L. The taxonomic position of Saccharomyces boulardii as evaluated by sequence analysis of the D1/D2 domain of 26S rDNA, the ITS1-5.8S rDNA-ITS2 region and the mitochondrial cytochrome-c oxidase II gene. Syst.Appl.Microbiol. 2003;26:564-571.

128. Fietto JL, Araujo RS, Valadao FN et al. Molecular and physiological comparisons between Saccharomyces cerevisiae and Saccharomyces boulardii. Can.J.Microbiol. 2004;50:615-621.

129. Guslandi M, Mezzi G, Sorghi M, Testoni PA. Saccharomyces boulardii in maintenance treatment of Crohn's disease. Dig.Dis.Sci. 2000;45:1462-1464.

130. Plein K, Hotz J. Therapeutic effects of Saccharomyces boulardii on mild residual symptoms in a stable phase of Crohn's disease with special respect to chronic diarrhea--a pilot study. Z.Gastroenterol. 1993;31:129-134.

131. Guslandi M, Giollo P, Testoni PA. A pilot trial of Saccharomyces boulardii in ulcerative colitis. Eur.J.Gastroenterol.Hepatol. 2003;15:697-698.

132. Silverberg MS, Satsangi J, Ahmad T et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Can.J.Gastroenterol. 2005;19 Suppl A:5-36.

133. D'Haens G, Sandborn WJ, Feagan BG et al. A review of activity indices and efficacy end points for clinical trials of medical therapy in adults with ulcerative colitis. Gastroenterology 2007;132:763-786.

134. Sandborn WJ, Feagan BG, Hanauer SB et al. A review of activity indices and efficacy endpoints for clinical trials of medical therapy in adults with Crohn's disease. Gastroenterology 2002;122:512-530.

135. Baumgart DC, Olivier WA, Reya T et al. Mechanisms of intestinal epithelial cell injury and colitis in interleukin 2 (IL2)-deficient mice. Cell Immunol. 1998;187:52-66.

136. Miltenyi S, Muller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. Cytometry 1990;11:231-238.

137. Dzionek A, Fuchs A, Schmidt P et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J.Immunol. 2000;165:6037-6046.

138. Chen X, Kokkotou EG, Mustafa N et al. Saccharomyces boulardii inhibits ERK1/2 mitogen-activated protein kinase activation both in vitro and in vivo and protects against Clostridium difficile toxin A-induced enteritis. J.Biol.Chem. 2006;281:24449-24454.

139. Hattori S, Hattori Y, Banba N, Kasai K, Shimoda S. Pentamethyl-hydroxychromane, vitamin E derivative, inhibits induction of nitric oxide synthase by bacterial lipopolysaccharide. Biochem.Mol.Biol.Int. 1995;35:177-183.

140. Mueller T, Terada T, Rosenberg IM, Shibolet O, Podolsky DK. Th2 cytokines down-regulate TLR expression and function in human intestinal epithelial cells. J.Immunol. 2006;176:5805-5814.

141. Suzuki M, Hisamatsu T, Podolsky DK. Gamma interferon augments the intracellular pathway for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular Toll-like receptor 4-MD-2 complex. Infect.Immun. 2003;71:3503-3511.

142. Hed J, Stendahl O. Differences in the ingestion mechanisms of IgG and C3b particles in phagocytosis by neutrophils. Immunology 1982;45:727-736.

143. Herzenberg LA, De Rosa SC. Monoclonal antibodies and the FACS: complementary tools for immunobiology and medicine. Immunol.Today 2000;21:383-390.

144. Baumgart DC, Metzke D, Schmitz J et al. Patients with active inflammatory bowel disease lack immature peripheral blood plasmacytoid and myeloid dendritic cells. Gut 2005;54:228-236.

145. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. J.Immunol.Methods 1994;171:131-137.

146. Lyons AB. Divided we stand: tracking cell proliferation with carboxyfluorescein diacetate succinimidyl ester. Immunol.Cell Biol. 1999;77:509-515.

147. Weston SA, Parish CR. New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. J.Immunol.Methods 1990;133:87-97.

148. Carson CF, Riley TV. Non-antibiotic therapies for infectious diseases. Commun.Dis.Intell. 2003;27 Suppl:S143-S146.

149. McLellan AD, Sorg RV, Williams LA, Hart DN. Human dendritic cells activate T lymphocytes via a CD40: CD40 ligand-dependent pathway. Eur.J.Immunol. 1996;26:1204-1210.

150. Young JW, Koulova L, Soergel SA et al. The B7/BB1 antigen provides one of several costimulatory signals for the activation of CD4+ T lymphocytes by human blood dendritic cells in vitro. J.Clin.Invest 1992;90:229-237.

151. Cella M, Scheidegger D, Palmer-Lehmann K et al. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J.Exp.Med. 1996;184:747-752.

152. Rogler G, Andus T. Cytokines in inflammatory bowel disease. World J.Surg. 1998;22:382-389.

153. Daig R, Andus T, Aschenbrenner E et al. Increased interleukin 8 expression in the colon mucosa of patients with inflammatory bowel disease. Gut 1996;38:216-222.

154. Struyf S, Gouwy M, Dillen C et al. Chemokines synergize in the recruitment of circulating neutrophils into inflamed tissue. Eur.J.Immunol. 2005;35:1583-1591.

155. Levings MK, Gregori S, Tresoldi E et al. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. Blood 2005;105:1162-1169.

156. Hoshino K, Takeuchi O, Kawai T et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J.Immunol. 1999;162:3749-3752.

157. Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J.Immunol. 2000;165:618-622.

158. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997;388:394-397.

159. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. Science 2001;294:1540-1543.

160. Cederblad B, Blomberg S, Vallin H et al. Patients with systemic lupus erythematosus have reduced numbers of circulating natural interferon-alpha- producing cells. J.Autoimmun. 1998;11:465-470.

161. Chehimi J, Campbell DE, Azzoni L et al. Persistent decreases in blood plasmacytoid dendritic cell number and function despite effective highly active antiretroviral therapy and increased blood myeloid dendritic cells in HIV-infected individuals. J.Immunol. 2002;168:4796-4801.

162. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. Cell 2001;106:255-258.

163. Sallusto F, Lanzavecchia A. Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. J.Exp.Med. 1999;189:611-614.

164. Kelsall BL, Leon F. Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease. Immunol.Rev. 2005;206:132-148.

165. Strober W, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. J.Clin.Invest 2007;117:514-521.

166. Laukoetter MG, Nava P, Nusrat A. Role of the intestinal barrier in inflammatory bowel disease. World J.Gastroenterol. 2008;14:401-407.

167. Shih DQ, Targan SR. Immunopathogenesis of inflammatory bowel disease. World J.Gastroenterol. 2008;14:390-400.

168. Ashcroft AJ, Cruickshank SM, Croucher PI et al. Colonic dendritic cells, intestinal inflammation, and T cell-mediated bone destruction are modulated by recombinant osteoprotegerin. Immunity. 2003;19:849-861.

169. Cruickshank SM, English NR, Felsburg PJ, Carding SR. Characterization of colonic dendritic cells in normal and colitic mice. World J.Gastroenterol. 2005;11:6338-6347.

170. Krajina T, Leithauser F, Moller P, Trobonjaca Z, Reimann J. Colonic lamina propria dendritic cells in mice with CD4+ T cell-induced colitis. Eur.J.Immunol. 2003;33:1073-1083.

171. Leithauser F, Trobonjaca Z, Moller P, Reimann J. Clustering of colonic lamina propria CD4(+) T cells to subepithelial dendritic cell aggregates precedes the development of colitis in a murine adoptive transfer model. Lab Invest 2001;81:1339-1349.

172. Malmstrom V, Shipton D, Singh B et al. CD134L expression on dendritic cells in the mesenteric lymph nodes drives colitis in T cell-restored SCID mice. J.Immunol. 2001;166:6972-6981.

173. Drakes ML, Blanchard TG, Czinn SJ. Colon lamina propria dendritic cells induce a proinflammatory cytokine response in lamina propria T cells in the SCID mouse model of colitis. J.Leukoc.Biol. 2005;78:1291-1300.

174. Ishiguro Y, Sakuraba H, Yamagata K, Munakata A. The presentation of haptenated proteins and activation of T cells in the mesenteric lymph nodes by dendritic cells in the TNBS colitis rat. Ann.N.Y.Acad.Sci. 2004;1029:346-347.

175. Karlis J, Penttila I, Tran TB et al. Characterization of colonic and mesenteric lymph node dendritic cell subpopulations in a murine adoptive transfer model of inflammatory bowel disease. Inflamm.Bowel.Dis. 2004;10:834-847.

176. Srikrishna G, Turovskaya O, Shaikh R et al. Carboxylated glycans mediate colitis through activation of NF-kappa B. J.Immunol. 2005;175:5412-5422.

177. O'Doherty U, Peng M, Gezelter S et al. Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. Immunology 1994;82:487-493.

178. Robinson SP, Patterson S, English N et al. Human peripheral blood contains two distinct lineages of dendritic cells. Eur.J.Immunol. 1999;29:2769-2778.

179. Rissoan MC, Soumelis V, Kadowaki N et al. Reciprocal control of T helper cell and dendritic cell differentiation. Science 1999;283:1183-1186.

180. Olweus J, BitMansour A, Warnke R et al. Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. Proc.Natl.Acad.Sci.U.S.A 1997;94:12551-12556.

181. Ikeda Y, Akbar F, Matsui H, Onji M. Characterization of antigen-presenting dendritic cells in the peripheral blood and colonic mucosa of patients with ulcerative colitis. Eur.J.Gastroenterol.Hepatol. 2001;13:841-850.

182. Ikeda Y, Akbar SM, Matsui H, Onji M. Antigen-presenting dendritic cells in ulcerative colitis. J.Gastroenterol. 2002;37 Suppl 14:53-55.

183. Vuckovic S, Florin TH, Khalil D et al. CD40 and CD86 upregulation with divergent CMRF44 expression on blood dendritic cells in inflammatory bowel diseases. Am.J.Gastroenterol. 2001;96:2946-2956.

184. Niess JH, Reinecker HC. Dendritic cells: the commanders-in-chief of mucosal immune defenses. Curr.Opin.Gastroenterol. 2006;22:354-360.

185. Rescigno M, Urbano M, Valzasina B et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat.Immunol. 2001;2:361-367.

186. te Velde AA, van KY, Braat H et al. Increased expression of DC-SIGN+IL-12+IL-18+ and CD83+IL-12-IL-18- dendritic cell populations in the colonic mucosa of patients with Crohn's disease. Eur.J.Immunol. 2003;33:143-151.

187. Watanabe S, Yamakawa M, Hiroaki T, Kawata S, Kimura O. Correlation of dendritic cell infiltration with active crypt inflammation in ulcerative colitis. Clin.Immunol. 2007;122:288-297.

188. Hart AL, Al-Hassi HO, Rigby RJ et al. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. Gastroenterology 2005;129:50-65.

189. Cong Y, Weaver CT, Lazenby A, Elson CO. Colitis induced by enteric bacterial antigen-specific CD4+ T cells requires CD40-CD40 ligand interactions for a sustained increase in mucosal IL-12. J.Immunol. 2000;165:2173-2182.

190. Stuber E, Strober W, Neurath M. Blocking the CD40L-CD40 interaction in vivo specifically prevents the priming of T helper 1 cells through the inhibition of interleukin 12 secretion. J.Exp.Med. 1996;183:693-698.

191. Battaglia E, Biancone L, Resegotti A et al. Expression of CD40 and its ligand, CD40L, in intestinal lesions of Crohn's disease. Am.J.Gastroenterol. 1999;94:3279-3284.

192. Gelbmann CM, Leeb SN, Vogl D et al. Inducible CD40 expression mediates NFkappaB activation and cytokine secretion in human colonic fibroblasts. Gut 2003;52:1448-1456.

193. Liu Z, Colpaert S, D'Haens GR et al. Hyperexpression of CD40 ligand (CD154) in inflammatory bowel disease and its contribution to pathogenic cytokine production. J.Immunol. 1999;163:4049-4057.

194. Vogel JD, West GA, Danese S et al. CD40-mediated immune-nonimmune cell interactions induce mucosal fibroblast chemokines leading to T-cell transmigration. Gastroenterology 2004;126:63-80.

195. MacDonald KP, Munster DJ, Clark GJ et al. Characterization of human blood dendritic cell subsets. Blood 2002;100:4512-4520.

196. Steinman RM, Nussenzweig MC. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. Proc.Natl.Acad.Sci.U.S.A 2002;99:351-358.

197. Hellman P, Eriksson H. Early activation markers of human peripheral dendritic cells. Hum.Immunol. 2007;68:324-333.

198. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. Nat.Med. 1999;5:1249-1255.

199. Jonuleit H, Kuhn U, Muller G et al. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. Eur.J.Immunol. 1997;27:3135-3142.

200. Roake JA, Rao AS, Morris PJ et al. Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. J.Exp.Med. 1995;181:2237-2247.

201. Banchereau J, Pascual V. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. Immunity. 2006;25:383-392.

202. Hawiger D, Inaba K, Dorsett Y et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J.Exp.Med. 2001;194:769-779.

203. O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity. 1998;8:275-283.

204. Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. Nat.Rev.Immunol. 2002;2:151-161.

205. Weaver DJ, Jr., Poligone B, Bui T et al. Dendritic cells from nonobese diabetic mice exhibit a defect in NF-kappa B regulation due to a hyperactive I kappa B kinase. J.Immunol. 2001;167:1461-1468.

206. Martin B, Banz A, Bienvenu B et al. Suppression of CD4+ T lymphocyte effector functions by CD4+CD25+ cells in vivo. J.Immunol. 2004;172:3391-3398.

207. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat.Clin.Pract.Gastroenterol.Hepatol. 2006;3:390-407.

208. Baumgart DC, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet 2007;369:1641-1657.

209. Kaplan GG, Hur C, Korzenik J, Sands BE. Infliximab dose escalation vs. initiation of adalimumab for loss of response in Crohn's disease: a cost-effectiveness analysis. Aliment.Pharmacol.Ther. 2007;26:1509-1520.

210. Panes J, Gomollon F, Taxonera C et al. Crohn's disease: a review of current treatment with a focus on biologics. Drugs 2007;67:2511-2537.

211. Boonstra A, sselin-Paturel C, Gilliet M et al. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. J.Exp.Med. 2003;197:101-109.

212. Langenkamp A, Messi M, Lanzavecchia A, Sallusto F. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat.Immunol. 2000;1:311-316.

213. Pulendran B, Smith JL, Caspary G et al. Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. Proc.Natl.Acad.Sci.U.S.A 1999;96:1036-1041.

214. Ida JA, Shrestha N, Desai S et al. A whole blood assay to assess peripheral blood dendritic cell function in response to Toll-like receptor stimulation. J.Immunol.Methods 2006;310:86-99.

215. Nikolaus S, Bauditz J, Gionchetti P et al. Increased secretion of pro-inflammatory cytokines by circulating polymorphonuclear neutrophils and regulation by interleukin 10 during intestinal inflammation. Gut 1998;42:470-476.

216. MacDonald TT, Hutchings P, Choy MY, Murch S, Cooke A. Tumour necrosis factor-alpha and interferon-gamma production measured at the single cell level in normal and inflamed human intestine. Clin.Exp.Immunol. 1990;81:301-305.

217. Murch SH, Braegger CP, Walker-Smith JA, MacDonald TT. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. Gut 1993;34:1705-1709.

218. Visintin A, Mazzoni A, Spitzer JH et al. Regulation of Toll-like receptors in human monocytes and dendritic cells. J.Immunol. 2001;166:249-255.

219. Winzler C, Rovere P, Zimmermann VS et al. Checkpoints and functional stages in DC maturation. Adv.Exp.Med.Biol. 1997;417:59-64.

220. Spoettl T, Hausmann M, Klebl F et al. Serum soluble TNF receptor I and II levels correlate with disease activity in IBD patients. Inflamm.Bowel.Dis. 2007;13:727-732.

221. Weber CR, Turner JR. Inflammatory bowel disease: is it really just another break in the wall? Gut 2007;56:6-8.

222. Radstake TR, van Lent PL, Pesman GJ et al. High production of proinflammatory and Th1 cytokines by dendritic cells from patients with rheumatoid arthritis, and down regulation upon FcgammaR triggering. Ann.Rheum.Dis. 2004;63:696-702.

223. Robak E, Smolewski P, Wozniacka A et al. Relationship between peripheral blood dendritic cells and cytokines involved in the pathogenesis of systemic lupus erythematosus. Eur.Cytokine Netw. 2004;15:222-230.

224. Mahida YR, Ceska M, Effenberger F et al. Enhanced synthesis of neutrophil-activating peptide-1/interleukin-8 in active ulcerative colitis. Clin.Sci.(Lond) 1992;82:273-275.

225. Seitz M, Dewald B, Ceska M, Gerber N, Baggiolini M. Interleukin-8 in inflammatory rheumatic diseases: synovial fluid levels, relation to rheumatoid factors, production by mononuclear cells, and effects of gold sodium thiomalate and methotrexate. Rheumatol.Int. 1992;12:159-164.

226. Mudter J, Neurath MF. Il-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. Inflamm.Bowel.Dis. 2007;13:1016-1023.

227. Feghali CA, Bost KL, Boulware DW, Levy LS. Mechanisms of pathogenesis in scleroderma. I. Overproduction of interleukin 6 by fibroblasts cultured from affected skin sites of patients with scleroderma. J.Rheumatol. 1992;19:1207-1211.

228. Hirano T. Interleukin-6 and its relation to inflammation and disease. Clin.Immunol.Immunopathol. 1992;62:S60-S65.

229. Tan PL, Farmiloe S, Yeoman S, Watson JD. Expression of the interleukin 6 gene in rheumatoid synovial fibroblasts. J.Rheumatol. 1990;17:1608-1612.

230. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J.Biol.Chem. 2003;278:1910-1914.

231. Harrington LE, Hatton RD, Mangan PR et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat.Immunol. 2005;6:1123-1132.

232. Langrish CL, Chen Y, Blumenschein WM et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J.Exp.Med. 2005;201:233-240.

233. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity. 2006;24:179-189.

234. Drakesmith H, O'Neil D, Schneider SC et al. In vivo priming of T cells against cryptic determinants by dendritic cells exposed to interleukin 6 and native antigen. Proc.Natl.Acad.Sci.U.S.A 1998;95:14903-14908.

235. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 2003;299:1033-1036.

236. Parronchi P, Romagnani P, Annunziato F et al. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. Am.J.Pathol. 1997;150:823-832.

237. Strober W, Kelsall B, Fuss I et al. Reciprocal IFN-gamma and TGF-beta responses regulate the occurrence of mucosal inflammation. Immunol.Today 1997;18:61-64.

238. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J.Immunol. 1991;147:3815-3822.

239. Mahida YR, Wu K, Jewell DP. Enhanced production of interleukin 1-beta by mononuclear cells isolated from mucosa with active ulcerative colitis of Crohn's disease. Gut 1989;30:835-838.

240. Kucharzik T, Stoll R, Lugering N, Domschke W. Circulating antiinflammatory cytokine IL-10 in patients with inflammatory bowel disease (IBD). Clin.Exp.Immunol. 1995;100:452-456.

241. Howard M, Muchamuel T, Andrade S, Menon S. Interleukin 10 protects mice from lethal endotoxemia. J.Exp.Med. 1993;177:1205-1208.

242. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell 1993;75:263-274.

243. Schreiber S, Heinig T, Thiele HG, Raedler A. Immunoregulatory role of interleukin 10 in patients with inflammatory bowel disease. Gastroenterology 1995;108:1434-1444.

244. Niess JH, Brand S, Gu X et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science 2005;307:254-258.

245. Franchimont D, Vermeire S, El HH et al. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. Gut 2004;53:987-992.

246. Michelsen KS, Aicher A, Mohaupt M et al. The role of toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCS). Peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. J.Biol.Chem. 2001;276:25680-25686.

247. Morath S, Stadelmaier A, Geyer A, Schmidt RR, Hartung T. Synthetic lipoteichoic acid from Staphylococcus aureus is a potent stimulus of cytokine release. J.Exp.Med. 2002;195:1635-1640.

248. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J.Biol.Chem. 1999;274:17406-17409.

249. Muzio M, Bosisio D, Polentarutti N et al. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J.Immunol. 2000;164:5998-6004.

250. Zhou H, Ding G, Liu W et al. Lipopolysaccharide could be internalized into human peripheral blood mononuclear cells and elicit TNF-alpha release, but not via the pathway of toll-like receptor 4 on the cell surface. Cell Mol.Immunol. 2004;1:373-377.

251. Ortega-Cava CF, Ishihara S, Rumi MA et al. Strategic compartmentalization of Toll-like receptor 4 in the mouse gut. J.Immunol. 2003;170:3977-3985.

252. Hausmann M, Kiessling S, Mestermann S et al. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology 2002;122:1987-2000.

253. Rigothier MC, Maccario J, Gayral P. Inhibitory activity of saccharomyces yeasts on the adhesion of Entamoeba histolytica trophozoites to human erythrocytes in vitro. Parasitol.Res. 1994;80:10-15.

254. Tasteyre A, Barc MC, Karjalainen T, Bourlioux P, Collignon A. Inhibition of in vitro cell adherence of Clostridium difficile by Saccharomyces boulardii. Microb.Pathog. 2002;32:219-225.

255. Berg R, Bernasconi P, Fowler D, Gautreaux M. Inhibition of Candida albicans translocation from the gastrointestinal tract of mice by oral administration of Saccharomyces boulardii. J.Infect.Dis. 1993;168:1314-1318.

256. Geyik MF, Aldemir M, Hosoglu S et al. The effects of Saccharomyces boulardii on bacterial translocation in rats with obstructive jaundice. Ann.R.Coll.Surg.Engl. 2006;88:176-180.

257. Castagliuolo I, Riegler MF, Valenick L, LaMont JT, Pothoulakis C. Saccharomyces boulardii protease inhibits the effects of Clostridium difficile toxins A and B in human colonic mucosa. Infect.Immun. 1999;67:302-307.

258. Girard P, Pansart Y, Coppe MC, Gillardin JM. Saccharomyces boulardii inhibits water and electrolytes changes induced by castor oil in the rat colon. Dig.Dis.Sci. 2005;50:2183-2190.

259. Pothoulakis C, Kelly CP, Joshi MA et al. Saccharomyces boulardii inhibits Clostridium difficile toxin A binding and enterotoxicity in rat ileum. Gastroenterology 1993;104:1108-1115.

260. Brandao RL, Castro IM, Bambirra EA et al. Intracellular signal triggered by cholera toxin in Saccharomyces boulardii and Saccharomyces cerevisiae. Appl.Environ.Microbiol. 1998;64:564-568.

261. Czerucka D, Roux I, Rampal P. Saccharomyces boulardii inhibits secretagogue-mediated adenosine 3',5'-cyclic monophosphate induction in intestinal cells. Gastroenterology 1994;106:65-72.

262. Czerucka D, Rampal P. Effect of Saccharomyces boulardii on cAMP- and Ca2+ -dependent Cl- secretion in T84 cells. Dig.Dis.Sci. 1999;44:2359-2368.

263. Dahan S, Dalmasso G, Imbert V et al. Saccharomyces boulardii interferes with enterohemorrhagic Escherichia coli-induced signaling pathways in T84 cells. Infect.Immun. 2003;71:766-773.

264. Schroeder B, Winckler C, Failing K, Breves G. Studies on the time course of the effects of the probiotic yeast Saccharomyces boulardii on electrolyte transport in pig jejunum. Dig.Dis.Sci. 2004;49:1311-1317.

265. Dalmasso G, Alexander G, Carlson H et al. Saccharomyces boulardii prevents and ameliorates trinitrobenzene sulfonic acid-induced colitis in mice. Gastroenterology 2005;128:A618.

266. Jawhara S, Poulain D. Saccharomyces boulardii decreases inflammation and intestinal colonization by Candida albicans in a mouse model of chemically-induced colitis. Med.Mycol. 20071-10.

267. Parkin J, Cohen B. An overview of the immune system. Lancet 2001;357:1777-1789.

268. Buts JP, Dekeyser N, Stilmant C et al. Saccharomyces boulardii produces in rat small intestine a novel protein phosphatase that inhibits Escherichia coli endotoxin by dephosphorylation. Pediatr.Res. 2006;60:24-29.

269. Buts JP, Bernasconi P, Vaerman JP, Dive C. Stimulation of secretory IgA and secretory component of immunoglobulins in small intestine of rats treated with Saccharomyces boulardii. Dig.Dis.Sci. 1990;35:251-256.

270. Rodrigues AC, Cara DC, Fretez SH et al. Saccharomyces boulardii stimulates sIgA production and the phagocytic system of gnotobiotic mice. J.Appl.Microbiol. 2000;89:404-414.

271. Akisu M, Baka M, Yalaz M, Huseyinov A, Kultursay N. Supplementation with Saccharomyces boulardii ameliorates hypoxia/reoxygenation-induced necrotizing enterocolitis in young mice. Eur.J.Pediatr.Surg. 2003;13:319-323.

272. Dalmasso G, Loubat A, Dahan S et al. Saccharomyces boulardii prevents TNF-alpha-induced apoptosis in EHEC-infected T84 cells. Res.Microbiol. 2006;157:456-465.

273. Sougioultzis S, Simeonidis S, Bhaskar KR et al. Saccharomyces boulardii produces a soluble anti-inflammatory factor that inhibits NF-kappaB-mediated IL-8 gene expression. Biochem.Biophys.Res.Commun. 2006;343:69-76.

274. Isolauri E, Kirjavainen PV, Salminen S. Probiotics: a role in the treatment of intestinal infection and inflammation? Gut 2002;50 Suppl 3:III54-III59.

275. Grijo NN, Borra RC, Sdepanian VL. Proinflammatory and Anti-inflammatory Cytokines Present in the Acute Phase of Experimental Colitis Treated with Saccharomyces boulardii. Dig.Dis.Sci. 2009

276. Neurath MF, Fuss I, Kelsall BL et al. Experimental granulomatous colitis in mice is abrogated by induction of TGF-beta-mediated oral tolerance. J.Exp.Med. 1996;183:2605-2616.

277. Manickasingham SP, Edwards AD, Schulz O, Reis e Sousa. The ability of murine dendritic cell subsets to direct T helper cell differentiation is dependent on microbial signals. Eur.J.Immunol. 2003;33:101-107.

278. Palucka K, Banchereau J. How dendritic cells and microbes interact to elicit or subvert protective immune responses. Curr.Opin.Immunol. 2002;14:420-431.

279. Milling SW, Yrlid U, Jenkins C et al. Regulation of intestinal immunity: effects of the oral adjuvant Escherichia coli heat-labile enterotoxin on migrating dendritic cells. Eur.J.Immunol. 2007;37:87-99.

280. Scheinecker C, McHugh R, Shevach EM, Germain RN. Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. J.Exp.Med. 2002;196:1079-1090.

281. Worbs T, Bode U, Yan S et al. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. J.Exp.Med. 2006;203:519-527.

282. Rimoldi M, Chieppa M, Salucci V et al. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat.Immunol. 2005;6:507-514.

283. Milling SW, Jenkins CD, Yrlid U et al. Steady-state migrating intestinal dendritic cells induce potent inflammatory responses in naive CD4+ T cells. Mucosal.Immunol. 2009;2:156-165.

284. Nussenzweig MC, Steinman RM, Witmer MD, Gutchinov B. A monoclonal antibody specific for mouse dendritic cells. Proc.Natl.Acad.Sci.U.S.A 1982;79:161-165.

285. McGovern D, Powrie F. The IL23 axis plays a key role in the pathogenesis of IBD. Gut 2007;56:1333-1336.

286. Hue S, Ahern P, Buonocore S et al. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. J.Exp.Med. 2006;203:2473-2483.

287. Kullberg MC, Jankovic D, Feng CG et al. IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. J.Exp.Med. 2006;203:2485-2494.

288. Uhlig HH, McKenzie BS, Hue S et al. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. Immunity. 2006;25:309-318.

289. Yen D, Cheung J, Scheerens H et al. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. J.Clin.Invest 2006;116:1310-1316.

290. Cua DJ, Sherlock J, Chen Y et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 2003;421:744-748.

291. Murphy CA, Langrish CL, Chen Y et al. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J.Exp.Med. 2003;198:1951-1957.

292. Williams DL, McNamee RB, Jones EL et al. A method for the solubilization of a (1----3)-beta-D-glucan isolated from Saccharomyces cerevisiae. Carbohydr.Res. 1991;219:203-213.

293. Yiannikouris A, Francois J, Poughon L et al. Alkali extraction of beta-d-glucans from Saccharomyces cerevisiae cell wall and study of their adsorptive properties toward zearalenone. J.Agric.Food Chem. 2004;52:3666-3673.

Table of figures

Table directory

Appendix

Table 15: Calculation of the modified Truelove Witts severity index (MTWSI) for ulcerative colitis. UC patients who scored ≥10 on the MTWSI were classified to have an active disease (flare up). The average MTWSI for patients in remission was 4.7 (range 0 - 9) and for flaring UC patients 11.6 (range 10 - 16); BM – bowel movement.

	Category	Score
A	Diarrhea	0 = 0-2 BM/day
		1 = 3-4 BM/day
		2 = 5-6 BM/day
		3 = 7-9 BM/day
		4 = 10 or more BM/day
B	Nocturnal diarrhea/ Early awakening for BM	0 = No
		1 = Yes
C	Bloody stools	0 = None
		1 = Occasionally with BM
		2 = 50% of BM
		3 = With every BM
D	Fecal incontinence/ soiling	0 = No
		1 = Yes
E	Abdominal pain/ cramping	0 = None
		1 = Mild; aware, but tolerable
		2 = Moderate; interferes with usual activities
		3 = Severe; incapacitating
F	General wellbeing	0 = Excellent
		1 = Very Good
		2 = Good
		3 = Fair
		4 = Poor
		5 = Terrible
G	Antidiarrheals/ narcotics	0 = No
		1 = Yes
H	Abdominal tenderness	0 = None
		1 = Mild to moderate; localised
		2 = Mild to moderate; diffuse
		3 = Severe or rebound tenderness

Table 16: Calculation of the Harvey Bradshaw severity index (HBSI) for Crohn`s Disease. CD patients who scored ≥7 on the HBSI were classified to have an active disease (flare up). The average HBSI for patients in remission was 2.8 (range 0 - 6.5) and flaring CD patients was 11 (range 7 - 22).

	Category	**Score**
A	General wellbeing	0 = Very well
		1 = Slightly illness
		2 = Poor
		3 = Very poor
		4 = Terrible
B	Abdominal pain	0 = None
		1 = Mild
		2 = Moderate
		3 = Severe
C	Number of liquid stools per day	Number of liquid stools
D	Abdominal mass	0 = None
		1 = Dubious
		2 = Definite
		3 = Definite and tender
E	Complications	Score 1 for each of them:
		Arthralgia
		Uveitis
		Erythema nodosum
		Pyoderma gangrenosum
		Aphthous ulcers
		Anal fissure
		New fistula
		Abscess

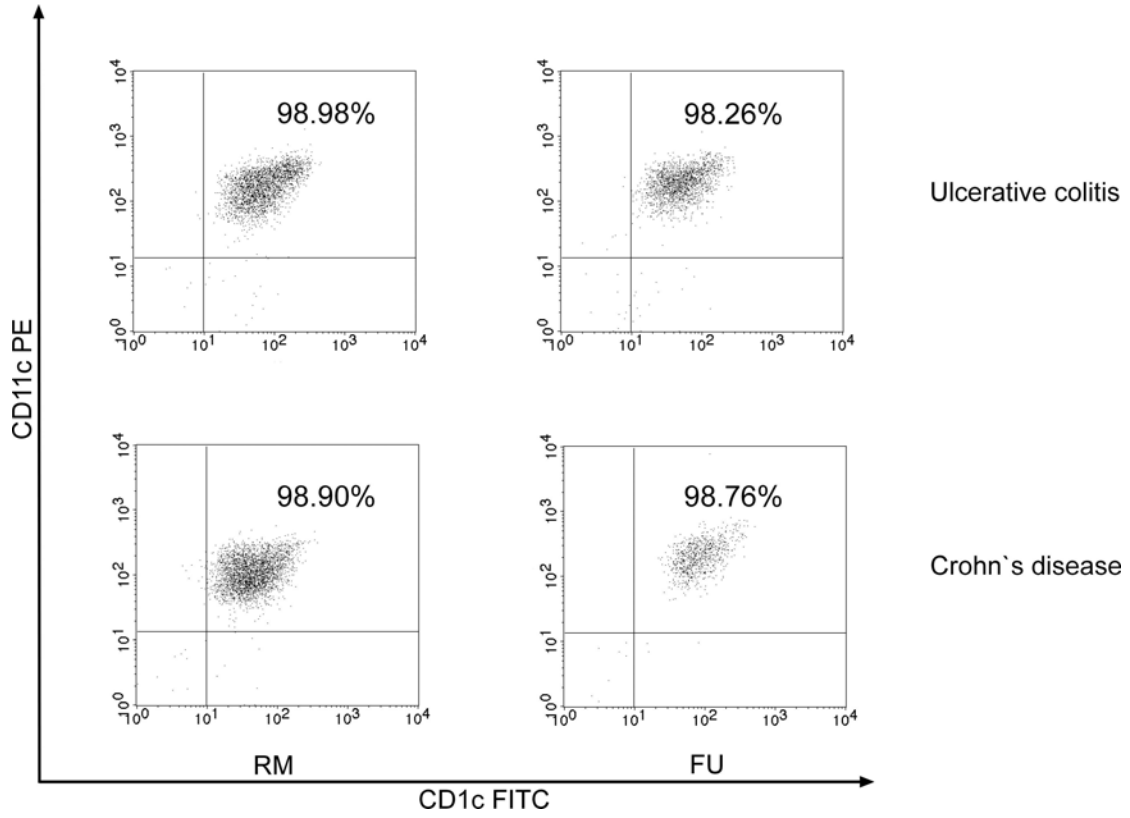

Figure 38: Isolation of human mDCs from IBD patients. Isolated mDCs were always $CD14^{-}CD19^{-}$ $CD11c^{+}CD1c^{+}$. FACS plots from representative experiments show staining for CD11c and CD1c. Quadrant thresholds were placed determined according to isotype controls. RM – Remission, FU – Flare-up.

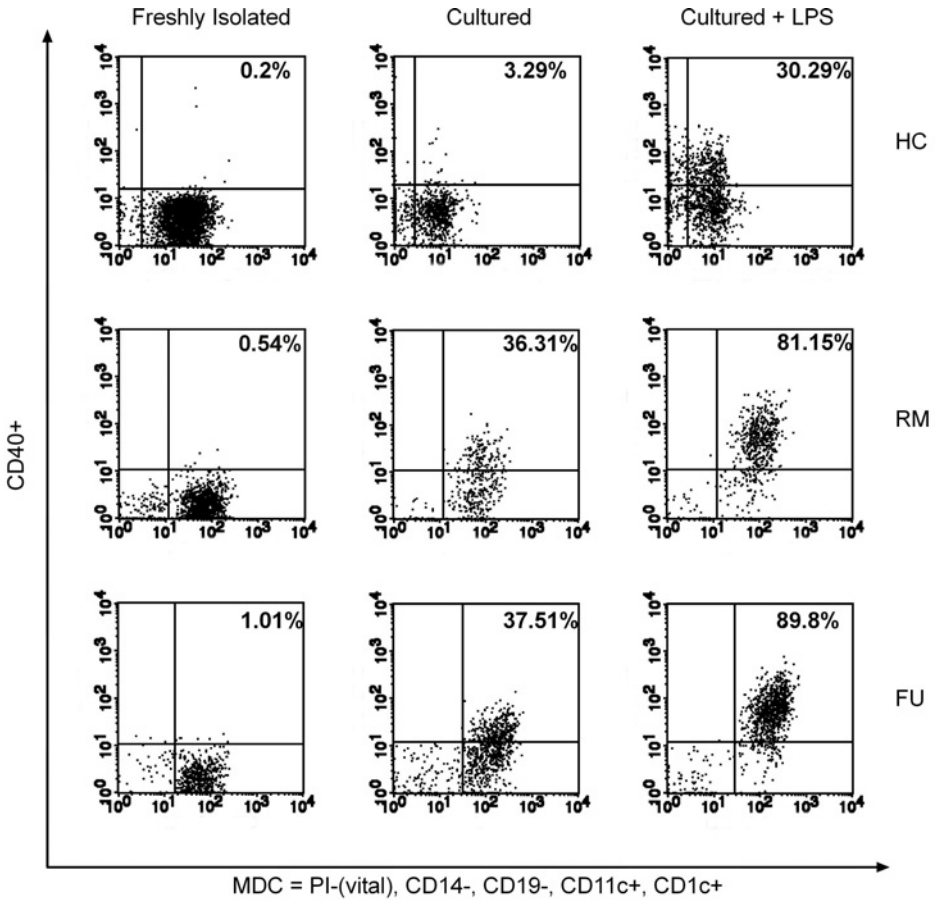

Figure 39: CD40 Expression in Ulcerative Colitis. More cultured and LPS stimulated, but not freshly isolated mDCs express CD40 in Ulcerative Colitis compared with healthy controls. Dot plots are representative for at least 6 independent experiments within each group. Quadrant thresholds were placed determined according to isotype controls. HC – Healthy Control, RM – Remission, FU – Flare-Up.

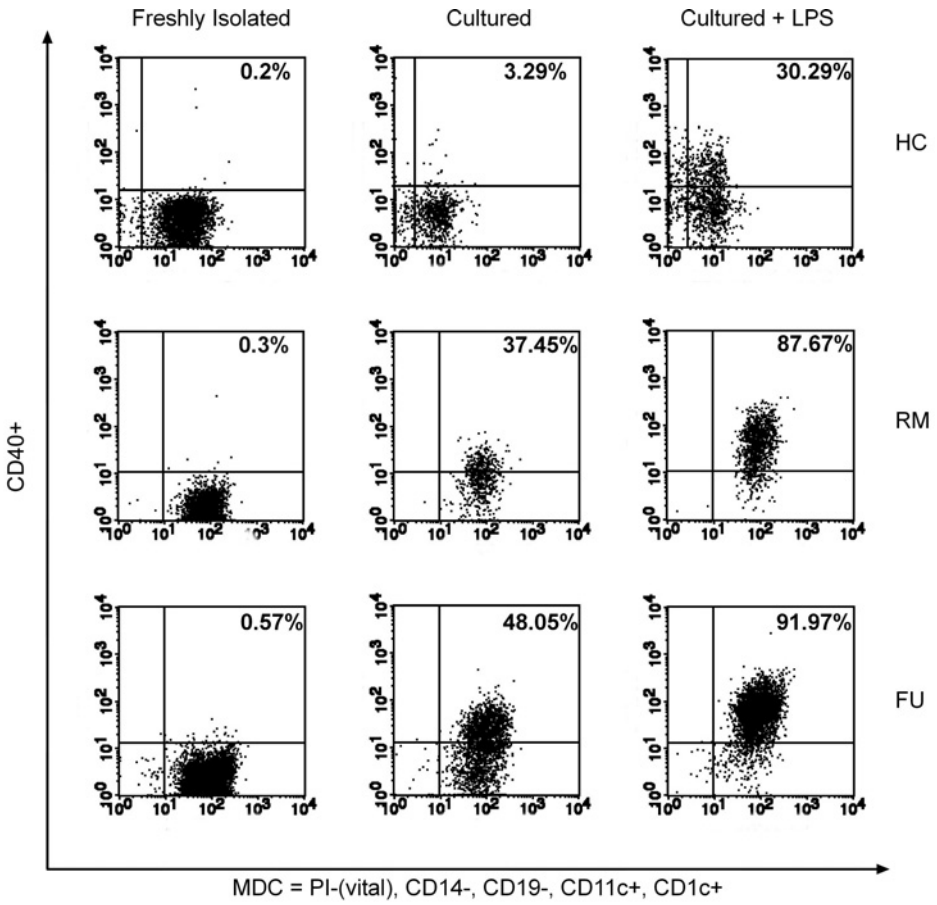

Figure 40: CD40 Expression in Crohn's Disease. More cultured and LPS stimulated, but not freshly isolated mDCs express CD40 in Crohn's Disease compared with healthy controls. Dot plots are representative for at least 6 independent experiments within each group. Quadrant thresholds were placed determined according to isotype controls. HC – Healthy Control, RM – Remission, FU – Flare-Up.

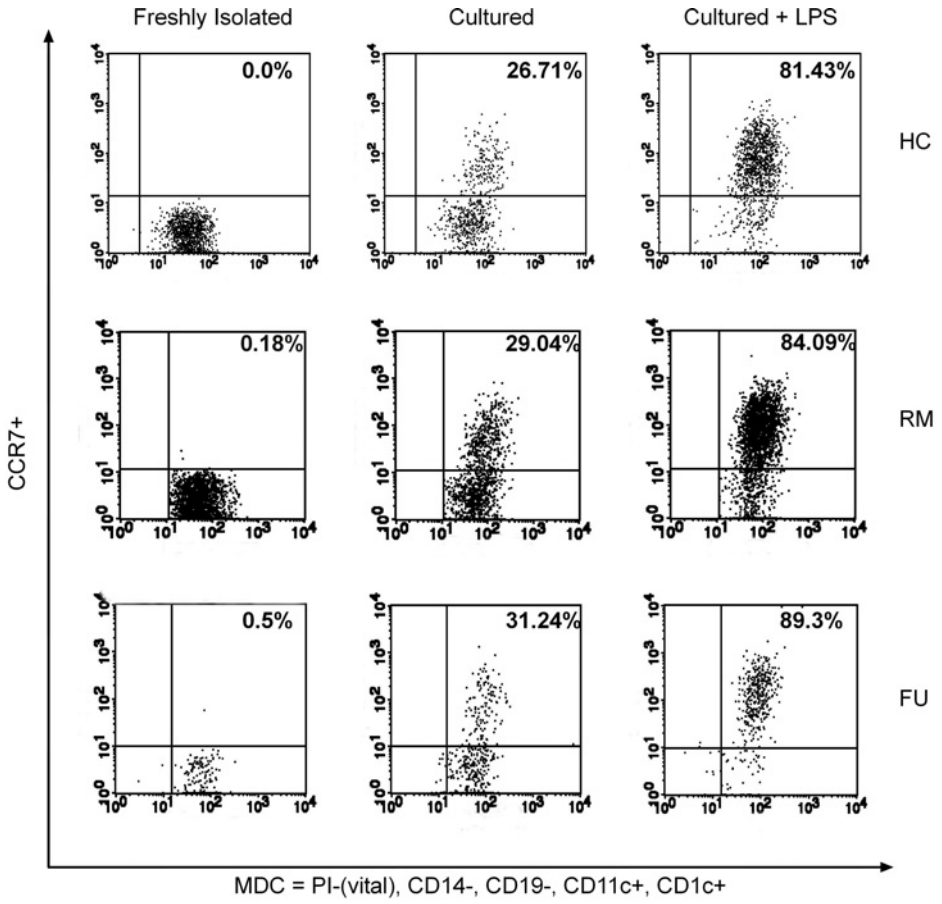

Figure 41: CD197 (CCR7) Expression in Ulcerative Colitis. More cultured and LPS stimulated, but not freshly isolated mDCs express CCR7 in healthy controls and in Ulcerative colitis. Dot plots show representatives for at least 5 independent experiments within each group. Quadrant thresholds were placed determined according to isotype controls. HC – Healthy Control, RM – Remission, FU – Flare-Up.

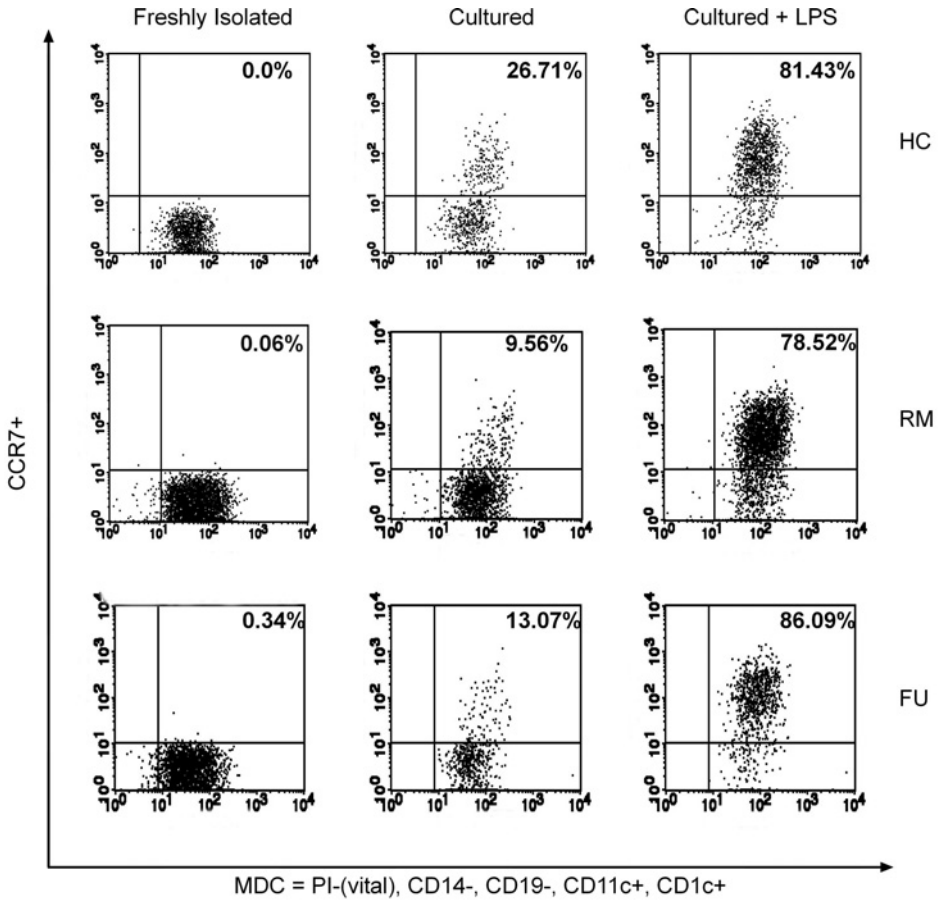

Figure 42: CD197 (CCR7) Expression in Crohn's Disease. More cultured and stimulated, but not freshly isolated mDCs express CCR7 in Crohn's Disease compared to Healthy Controls. Representative dot plots are shown. Quadrant tresholds were placed determined according to isotype controls. HC – Healthy Control, RM – Remission, FU – Flare-Up.

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List of publications

Baumgart DC, Metzke D, Guckelberger O, Pascher A, Grötzinger C, Przesdzing I, Dörffel Y, Schmitz J, Radbruch A, *Thomas S*. Aberrant plasmacytoid dendritic cell distribution and function in patients with Crohn's disease and ulcerative colitis. In press.

Baumgart DC, *Thomas S*, Przesdzing I, Metzke D, Bielecki C, Lehmann S, Lehnardt S, Dörffel Y, Sturm A, Scheffold A, Schmitz J, Radbruch A. Exaggerated inflammatory response of primary human myeloid dendritic cells to Lipopolysaccharide in patients with inflammatory bowel disease. Clin Exp Immunol. 2009 Sep;157(3):423-36.

Thomas S, Przesdzing I, Metzke D, Schmitz J, Radbruch A, Baumgart DC. *Saccharomyces boulardii* inhibits lipopolysaccharide induced activation of human dendritic cells and T-cell proliferation. Clin Exp Immunol. 2009 Apr;156(1):78-87. Epub 2009 Jan 21.

Tsuiji M, Yurasov S, Velinzon K, *Thomas S*, Nussenzweig MC, Wardemann H. A checkpoint for autoreactivity in human IgM+ memory B cell development. J Exp Med. 2006 Feb 20;203(2):393-400. Epub 2006 Jan 30.

Presented poster

Digestive Disease Week 2011, Chicago, USA.

Keystone Symposia 2011: Dendritic Cells and the Initiation of Adaptive Immunity. Santa Fe, USA.

Gastro 2009, UEGW/ WCOG London, UK.

2nd European Congress of Immunology, ECI 2009, Berlin, Germany.

Keystone Symposia 2009: Pattern Recognition Molecules and Immune Sensors of Pathogens (Z1), Banff, Canada.

Falk Intestinal Disease Meeting, 2006, Berlin, Germany.

Digestive Disease Week, DDW 2006, Los Angeles, U.S.A.

Ort:

Datum: Unterschrift:

Eidesstattliche Erklärung

Hiermit versichere ich, Saskia Thomas (geboren am 23.05.1980 in Berlin), an Eides statt, dass die vorliegende Arbeit mit dem Titel „Aberrant response of human myeloid dendritic cells to microbial stimuli in patients with inflammatory bowl disease“ selbstständig und ohne unerlaubte Hilfe angefertigt wurde. Alle verwendeten Hilfsmittel und Ausführungen aus anderen Quellen wurden als solche kenntlich gemacht. Zudem versichere ich, dass die vorliegende Arbeit noch nicht Gegenstand eines anderen Prüfungs- oder Promotionsverfahrens war. Weiterhin habe ich noch keinen Doktorgrad erlangt oder zu erlangen versucht. Die Inhalte der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin sind mir bekannt.

Ort:

Datum: Unterschrift: